Université de Sherbrooke

Cytogenetic and molecular investigation of terminal deletion of the long arm of chromosome 7 in three cases.

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List of Abbreviations

BrdUrd	5-**br**omo-2'-**d**eoxy**u**ridine
CGH	**C**omparative **G**enomic **H**ybridization
aCGH	**A**rray- **C**omparative **G**enomic **H**ybridization
DNA	**D**eoxyribo**N**ucleic **A**cid
FISH	**F**luorescent ***In Situ*** **H**ybridization
GTG	**G**-Bands by **T**rypsin digestion using **G**iemsa staining
HPE	**Holopros**encephaly
Mb	**Mega**base
m-Banding	**M**ulticolor-**B**anding
M-FISH	**M**ultiplex-FISH
PRINS	**P**rimed ***In Situ*** Labeling
PNA-FISH	**P**eptide **N**ucleic **A**cid- **F**luorescent ***In Situ*** **H**ybridization
SKY	**S**pectral **K**aryotype
SO	**S**pectrum **O**range
SG	**S**pectrum **G**reen
Sub-tel	**Sub-tel**omere
7q	Long arm of chromosome 7
7p	Short arm of chromosome 7

Summary

Chromosomal anomaly is an abnormality of the number or structure of the chromosome. Based on this, they are classified as either numerical or structural. These anomalies can have a mild or severe effect on the phenotype of the carrier which depends on the chromosomal region involved and the genes implicated. They can be sporadic or inherited. Thus, it is essential to investigate such anomalies both prenatally and postnatally alike. In a standard cytogenetic lab, these anomalies can be detected using low-resolution or high-resolution karyotype, usually by performing GTG (G-bands by trypsin using Giemsa) on chromosomes undergoing mitosis, derived from blood. If higher resolution is required, there are various other molecular cytogenetic techniques available like FISH and microsatellite analysis. More recently, microarrays have opened a new era in the field of molecular genetics by greatly increasing the resolution of screening for copy number gains and losses. Using these techniques, we characterized deletions in the long arm of chromosome 7 in three clinical cases, identified the breakpoint, studied the inheritance pattern and compared our cases with the other cases carrying similar deletion in the literature. It was observed that the deletion was located at 7q36.2, 7q35 and 7q36.1 for cases 1, 2 and 3 respectively. Case 3 also carried a duplication of Xq28.

Key Words

Chromosome 7 | Holoprosencephaly | Chromosome deletion

Partial monosomy 7q36 | Sacral agenesis

Preface

Chromosome abnormalities occur with a frequency estimated to be about 10-30% of all fertilized eggs. Of the different types of chromosome abnormalities, aneuploidy (trisomy and monosomy) is considered to be the most common and, clinically, the most important. Over 25% of miscarriages are due to monosomy or trisomy, making aneuploidy one of the leading causes of pregnancy loss (Hassold et al., 1996).

In addition, terminal deletion or partial monosomy of a chromosomal region often results in significant pathology like mental retardation, growth delay, cleft lip and palate etc. Therefore it is essential to investigate such cases and establish a genotype-phenotype correlation, such that proper diagnosis and care can be provided.

The main project during my Master's was investigation of three patients with deletion of the long arm of chromosome 7 (7q). Characterization of the deletion and its association with the patient's phenotype was done using cytogenetic and molecular genetic techniques. These techniques were performed by me except for the array-CGH (all cases), that was done at GeneDx.

I wrote the initial draft of the paper under the guidance of Mr. Macoura Gadji, who is the second author of the article.

This article has never been a part of any other thesis and is only included in my thesis, I being the first author.

Dr. Sylvie Cote and Dr. Kada Krabchi are the doctors who investigated patient 1. Dr. Bruno Maranda and Dr. J. Gekas are the doctors who clinically investigated the patients 2 and 3 in Quebec.

Addendum:

Before working on the three patients described above, I was involved in various parts of the fetal cell project for my training in the laboratory. The aim of the fetal cell project is to be able to detect aneuploidy in the fetus in a manner that is non-invasive, since the invasive techniques like amniocentesis and chorionic villus sampling pose a certain risk to the fetus. This is made possible by the presence of fetal cells found in maternal peripheral blood (Herzenberg et al., 1979; Walknowska et al., 1969). Quantification of these fetal cells by targeting XY cells using FISH and PRINS in normal and abnormal male pregnancies has been carried out in our lab by Dr. Kada Krabchi by manual scanning. It was observed that approximately 2-6 fetal cells are present per ml of maternal blood (Krabchi et al., 2001) and the number seemed to increase in cases of aneuploidy (Krabchi et al., 2006a; Krabchi et al., 2006b). Therefore, to spot these 2 to 6 cells (using Y as marker chromosome) among millions of XX cells, by manual scanning is a technical challenge.

Thus, we worked on validating Ikoniscope- an automatic slide scanning apparatus for detecting rare XY cells among thousands of XX cells. In this project, I did not work on real cases from pregnant women but simulated a similar condition by taking blood from normal male and female persons and harvesting it using standard procedure, spreading 1-

2 XY cells on clean slides under optimal conditions, counting, imaging and taking coordinates of these cells and finally spreading XX cells on top of them. This was followed by FISH with X in SO and Y in SG. The slides were then loaded on the Ikoniscope for automatic scanning and the pictures taken by the machine were analyzed and classified as true or false positive. The advantage of this protocol was that we already knew the number of XY cells on the slide and we could compare the XY cells spread by us and the number of cells retrieved by the Ikoniscope.

In the part described above, I worked on harvesting of blood, spreading XY and XX cells on slide, counting and imaging XY cells before spreading XX on top of it, cross checked by another student, doing FISH, analyzing pictures on Ikoniscope, going back to the cells missed by the Ikoniscope manually on the microscope and investigating the reason why the cells were missed.

Since manual scanning of rare events has never been validated we followed the same protocol for validation of manual scanning. The slides were scanned manually instead of automatically by me and another student in the lab.

Getting involved in this project taught me various techniques like, blood harvest, FISH, recognizing a FISH signal, differentiating between a false positive and a true positive and spreading very few (1-10) cells on a slide.

For validation of manual scanning, I was involved in counting and imaging XY cells prior to spreading XX cells cross counted by another student. I also manually scanned slides in this part.

Other goal of this project was to establish a technique that was the best for detecting rare events among FISH, PRINS and PNA-FISH. Consequently, this was followed by improvement of the PRINS technique for comparison with other techniques. I tried various different protocols to optimize the technique. Thus, it exposed me to different useful parameters of research.

1. INTRODUCTION

1.1) Definition and History

Genetics is the branch of biology that deals with the study of heredity. The hereditary nature of living organisms is defined by their genome which consists of nucleic acid. Deoxyribonucleic acid (DNA) is the raw material of inheritance in humans and is functionally divided into genes and produces proteins essential for life. A very long molecule of DNA along with the associated proteins comprises a chromosome. The study of structure, function and evolution of chromosomes is referred to as cytogenetics. Human cytogenetics had its beginning in the nineteenth century. The term "chromosome" was first introduced in 1888 which means colored body in Greek (Waldeyer 1888). The number of chromosomes in a normal human was corrected to 46 in 1956 (Tjio 1956), (Ford et al., 1956), it was considered to be 48 since 1923.

Soon clinical cytogenetics emerged. It deals with the cellular aspects of heredity, especially the chromosomes, to diagnose medical conditions (mental retardation, multiple malformations, cancer) caused by **genomic aberrations.** These aberrations are a result of an abnormal structure or number of chromosomes. The first chromosome abnormality described in human was Down syndrome. Lejeune et al. found that Down syndrome is caused by the presence of three copies of chromosome 21 (trisomy 21) instead of the usual two copies (Lejeune et al., 1959a; Lejeune et al., 1959b).

Detailed studies of human chromosomes became possible only after several technical developments. Peter Nowell in 1960, observed that the kidney bean extract phytohemagglutinin, stimulated lymphocytes to divide (Nowell 1960). Consequently chromosome studies could be carried out much more easily. The introduction of chromosome banding revolutionized human cytogenetics in the following years. Over the past 25 years molecular cytogenetic techniques of increasingly high-resolution have been developed.

1.2) Chromosome

1.2.1) Compaction and Packaging

A chromosome is made up of chromatin which is a highly structured complex of DNA and various histone and nonhistone proteins. The DNA is five to ten times more condensed in metaphase chromosomes than in interphase chromatin. The DNA double helix is 2 nm in diameter and is wound around proteins. Histones are low molecular weight basic proteins that have high-binding affinity for DNA. The DNA is wound around a protein disk consisting of histones H2A, H2B, H3, and H4. The centre of the disk consists of two molecules each of H3 and H4 and each of the faces consisting of an H2A-H2B complex. The DNA is wound twice around the histone octamer to form a **nucleosome core**. The two coils of DNA, containing 146 bp are compacted into 5.7 nm, which is the thickness of histone disk. Nucleosome cores are connected through DNA linkers of 90-100 bp. A nucleosome thus consists of the core and linker containing about 200 bp of DNA. The nucleosome represents the first level of packaging. When chromatin

is dispersed, the chromosome strands appear in electron micrograph as 11 nm disk like beads on a DNA string (***Figure 1***). Single molecules of histone 1 (H1) attach to the nucleosomes at the position of the linkers.

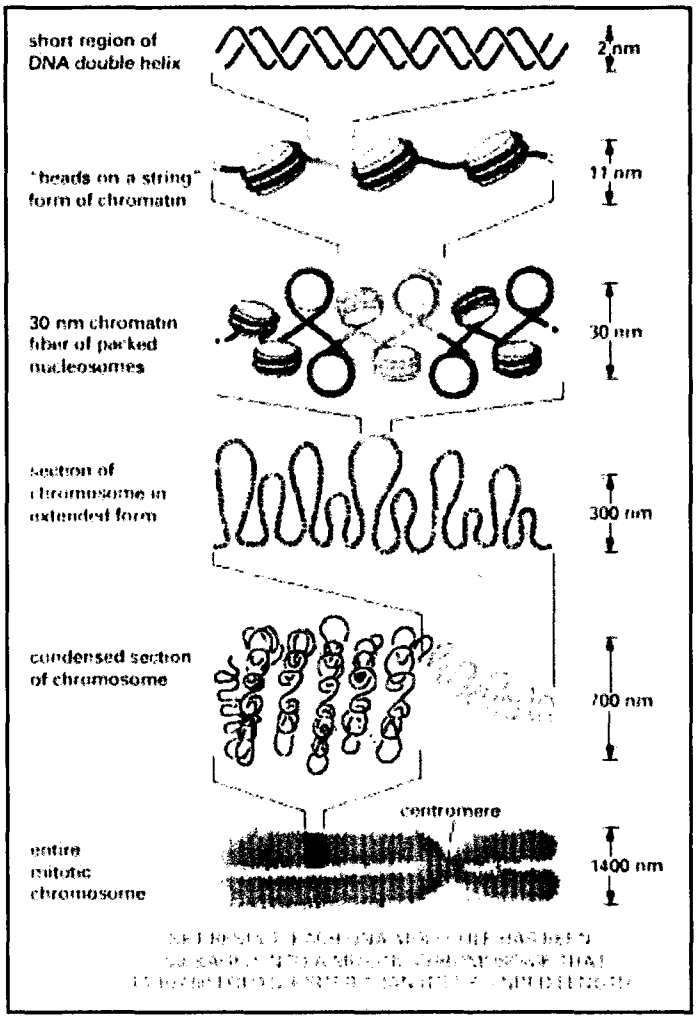


Figure 1: *Different levels of chromatin packaging (Figure 4-55, Alberts et al., Molecular Biology of the cell, 2002) (Alberts et al., 2002).*

This twists the successive nucleosome cores in to a 30-nm fiber, producing another six-fold DNA compaction. The next level of packing leads to the formation of thicker fiber,

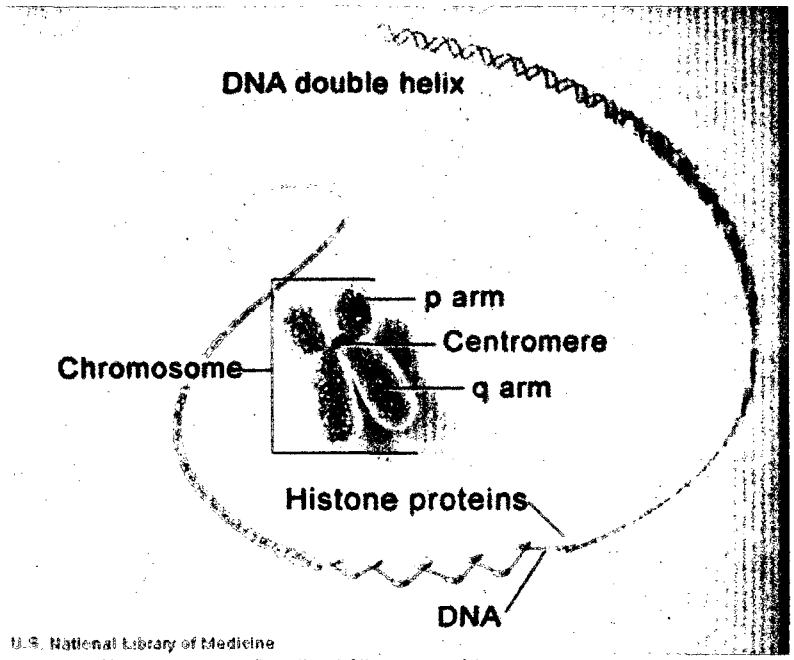
130-300 nm. This structure is made of loops, called domains. DNA and histones make up about two-third of the chromosome mass; the rest is made up by mostly nonhistone proteins. These proteins include various HMG (high-mobility-group) proteins, and a few rather insoluble scaffold proteins, which make up about 5% of the chromosome mass. Above the level of 30-nm fiber, chromatin is constrained by scaffold proteins into loop domains. Thereafter, several levels of structure are formed in a similar manner to a more condensed state, to yield the highest level of compaction, the metaphase chromosome.

1.2.2) Structure

A typical replicated metaphase chromosome consists of two sister chromatids ***(Figure 2),*** which becomes daughter chromosomes after its separation at anaphase. Each chromatid has two arms separated by a **centromere**, the site of spindle microtubule attachment. Mitotic spindle fibers are the functional elements that separate the sister chromatids during cell division. Each chromosome has a characteristic length (1-10μm or so) and position of the centromere which divides the chromosome into short arm, designated as p arm, and long arm, designated as q arm (Paris conference 1971) ***(Figure 2).*** The physical ends of the chromosome are capped by **telomeres** that contain short TTAGGG repeats and proteins. Telomeres protect the chromosome ends from end-to-end fusion.

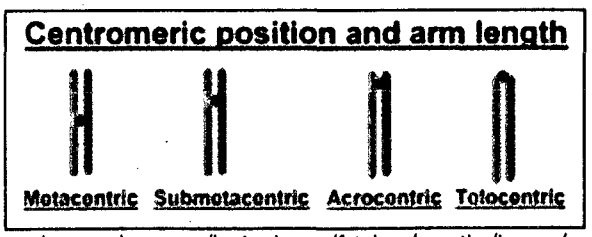
According to the position of the centromere and relative lengths of p and q arm, the chromosome can be classified as- metacentric, sub-metacentric, acrocentric, or telocentric ***(Figure 3)***. Metacentric chromosomes have the centromere near the middle,

submetacentric chromosomes have the centromere so placed that it divides the chromosome into two arms of unequal length (p arm being short and q being long), acrocentric chromosomes have it located quite near one end of the chromosome, and telocentric chromosomes have centromeres at the end. Telocentric chromosomes do not exist in normal humans and are found only as a result of a structural change.



http://ghr.nlm.nih.gov/handbook/illustrations/chromosomestructure.jpg

Figure 2: *A condensed metaphase chromosome with highly compact DNA coiled around proteins.*



http://www.obgynacademy.com/basicsciences/fetology/genetics/images/centpos.gif

Figure 3: *Metacentric, submetacentric, acrocentric and telocentric chromosomes.*

The study of chromosomes is important in understanding etiology of mental retardation, multiple malformation, cancer, infertility and spontaneous abortion which could be a manifestation of underlying genetic anomaly. It is therefore essential to study whether or not there are any kinds of genomic aberration involved.

1.3) Genomic Aberrations can be classified as follows-

1.3.1) Single gene disorders

1.3.2) Multifactorial disorders

1.3.3) Chromosome abnormality

1.3.1) Single gene disorders - These disorders are caused by mutations or defects in single gene e.g. – Thalassemias, Huntington disease.

1.3.2) Multifactorial disease - The disease is a result of a combination of small variations in genes that together produce a serious defect often concert with environmental factors and lifestyle e.g.- Diabetes type 2.

1.3.3) Chromosome abnormality is an anomaly in the normal structure or number of chromosome(s). It is of two types. If it reflects an abnormality in the chromosome number it is **Numerical,** while an aberration in the structure of the chromosome is called a **Structural anomaly**. These abnormalities usually occur when there is an error in cell division following meiosis or mitosis. They can also occur due to

several mutations caused by DNA damaging agents like UV rays. They can be acquired during lifetime (*de novo*) or inherited.

1.3.3.1) Numerical anomaly is classified as – **Aneuploidy** and **Polyploidy**.

1.3.3.1.1) Aneuploidy- The occurrence of one or more extra or missing chromosome(s) ***(Figure 4)*** leading to an unbalanced chromosome complement. It is mostly caused by an error in cell division. This mainly leads to trisomy or monosomy of the chromosome.

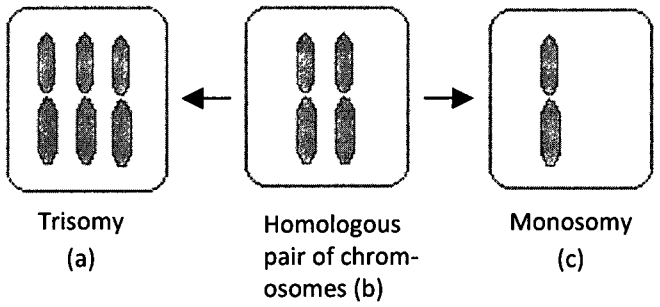
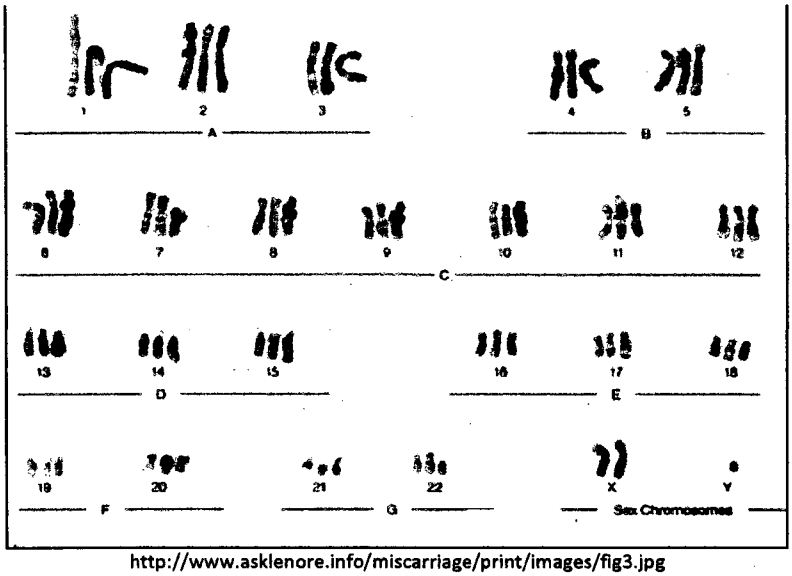


Figure 4: *A normal homologous pair of chromosomes (b), three copies of the chromosome (trisomy) (a), one copy of the chromosome (monosomy) (c).*

1.3.3.1.2) Polyploidy- Cells are polyploid if they contain more than two haploid (n) sets of chromosomes. For example, triploid (3n) ***(Figure 5)*** and tetraploid (4n) cells. Most polyploid embryos die early in pregnancy and are spontaneously aborted.

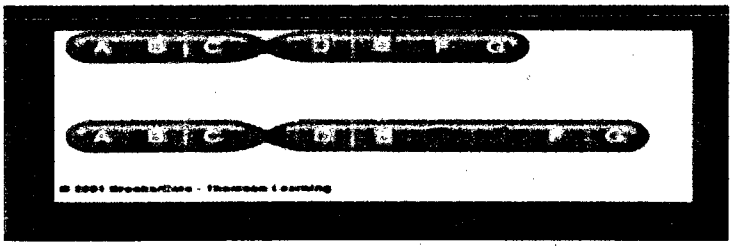


http://www.asklenore.info/miscarriage/print/images/fig3.jpg

Figure 5: *An extra copy of each chromosome is present resulting in an extra haploid set of chromosomes (triploidy).*

1.3.3.2) Structural anomaly is further classified as – **Insertion, duplication, inversion, ring, translocation** and **deletion.**

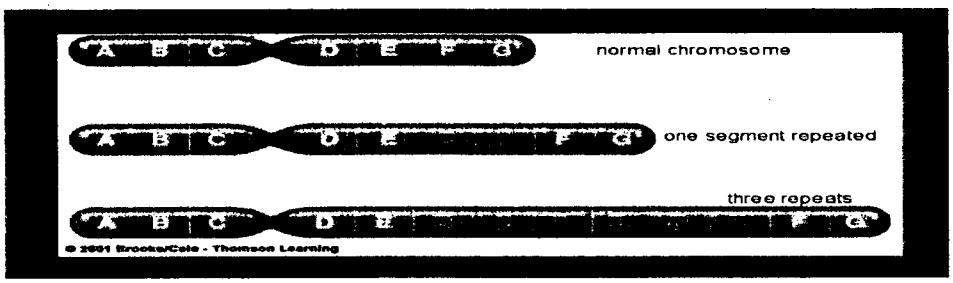
1.3.3.2)1. Insertion- It is the insertion of a chromosomal segment into another chromosome. This results in extra genetic material and can cause deleterious phenotypes in the carrier. The segments L, K have been inserted in between segments E and F ***(Figure 6)***.



http://trc.ucdavis.edu/biosci10v/bis10v/week4/duplicationpic.gif (modified)

Figure 6: *An Insertion.*

1.3.3.2)2. Duplication- A portion of the chromosome is duplicated, resulting in extra genetic material ***(Figure 7).*** The segments D, E are repeated once and thrice in the two chromosomes below. This results in extra chromosomal material.

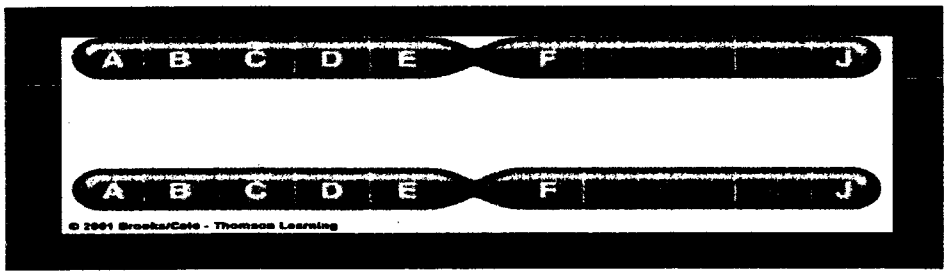


http://trc.ucdavis.edu/biosci10v/bis10v/week4/duplicationpic.gif

Figure 7: *A Duplication.*

1.3.3.2)3. Inversion- A portion of the chromosome breaks off, turns upside down and reattaches, thus the genetic material is inverted. The segment GHI, has been inverted to IHG ***(Figure 8)***. Inversions are either pericentric (centromere involved) or paracentric (centromere not involved).

Fig. 1.8 is an example of paracentric inversion.

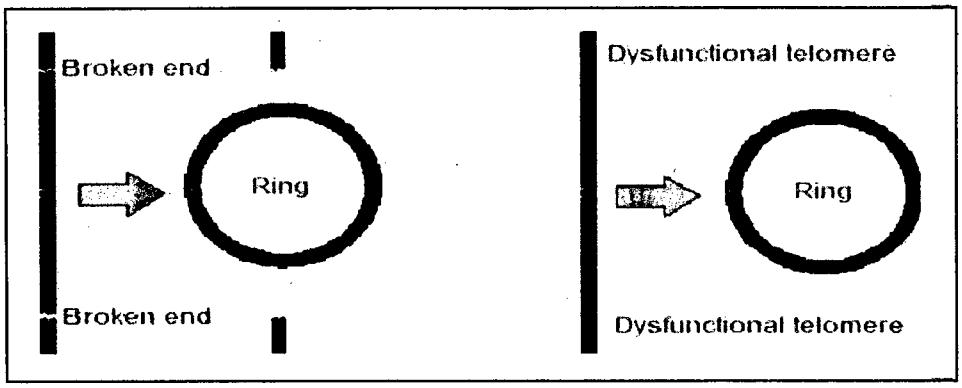


http://trc.ucdavis.edu/biosci10v/bis10v/week4/inversion.gif

Figure: 8: *An Inversion.*

1.3.3.2)4. Ring- A portion of the chromosome breaks off, the sticky ends come together and join to form a circle or ring.

Note the two chromosome ends break and the sticky ends join to form a ring chromosome in the figure below ***(Figure 9)***.

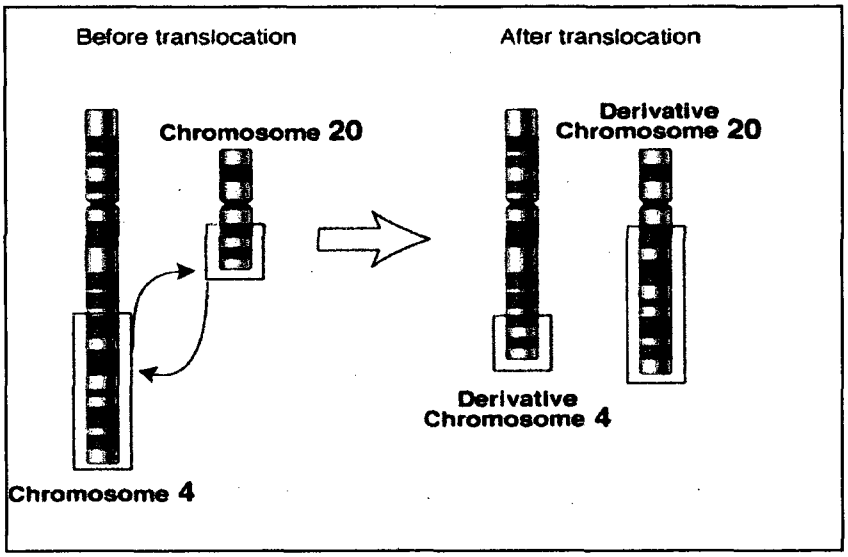


http://gurfein.com/cubby/images/rings.gif

Figure 9: *Ring chromosome.*

1.3.3.2)5. Translocation- A chromosome translocation is a chromosome abnormality caused by a rearrangement of parts between two non-homologous chromosomes. This is classified as **reciprocal** or **robertsonian.** Reciprocal is further divided as **balanced** and **unbalanced.**

1.3.3.2)5.1.1 Balanced reciprocal translocation- When there is no net loss or gain of genetic material due to a rearrangement between two non-homologous chromosomes, it is called a balanced translocation ***(Figure 10).***



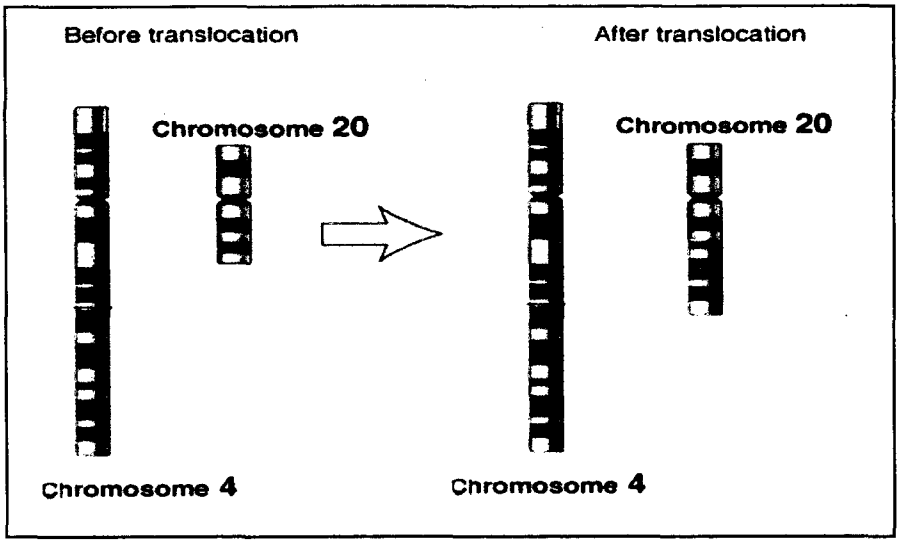
http://www.genome.gov/Pages/Hyperion/DIR/VIP/Glossary/Illustration/Images/translocation.gif

Figure 10: *A balanced translocation between chromosome 4 and 20.*

1.3.3.2)5.1.2 Unbalanced reciprocal translocation- When there is a net loss or gain of genetic material due to a rearrangement between two non-homologous chromosomes, it is called an unbalanced translocation. Shown in the figure is a reciprocal unbalanced translocation between chromosome 4 and 20. The extra genetic material on the derived chromosome 20 attributes to an unbalanced translocation. ***(Figure 11)***.

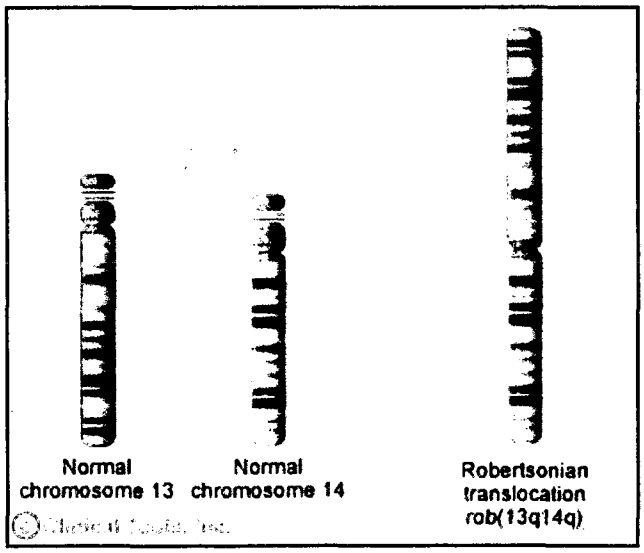
1.3.3.2).5.2 Robertsonian translocation- Robertsonian translocation is a common form of chromosomal rearrangement that occurs between two acrocentric chromosomes ***(Figure 12)***. It is formed by fusion of the whole

long arms of two acrocentric chromosomes. Breaks may also occur in one short arm and one long arm.



http://www.genome.gov/Pages/Hyperion/DIR/VIP/Glossary/Illustration/Images/translocation.gif (modified)

Figure 11: *An unbalanced reciprocal translocation.*

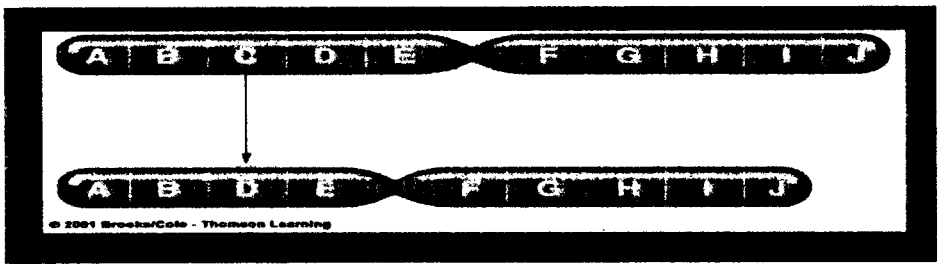


https://images2.clinicaltools.com/images/gene/13_14_derivative.jpg

Figure 12: *A robertsonian translocation.*

1.3.3.2)6. Deletion- Deletion is a structural anomaly where a part of the chromosome is lost/deleted resulting in loss of genetic material.

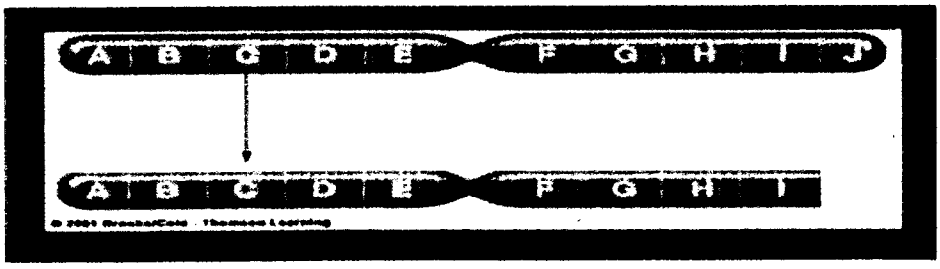
1.3.3.2)6.1. Interstitial deletions occur when a middle portion of the chromosome gets deleted. It arises if there are two breaks in the same arm of the chromosome. The segment C is deleted resulting in an interstitial deletion ***(Figure 13)***.



http://trc.ucdavis.edu/biosci10v/bis10v/week4/deletion.gif

Figure 13: *An interstitial deletion.*

1.3.3.2)6.2. If the loss of the chromosomal segment is due to a single break it results in a **terminal deletion.** The segment J is deleted at the end of the long arm of the chromosome giving rise to a terminal deletion ***(Figure 14)***.



http://trc.ucdavis.edu/biosci10v/bis10v/week4/deletion.gif (modified)

Figure 14: *A terminal deletion.*

These genetic aberrations are studied using following cytogenetic and molecular genetic tools.

1.4) **Standard cytogenetic techniques-** Conventional or standard cytogenetic techniques are the basic techniques used in laboratories for many years for chromosome analysis. These techniques have wide applications, from clinical diagnostics to basic genomic research.

1.4.1) **Cell Culture**- Actively dividing cells are required for studying chromosomes using traditional cytogenetic techniques. Specimens (blood, amniotic fluid, skin fibroblasts) for chromosome preparation are grown and maintained in an aqueous growth medium. For solid tissues like skin biopsy, the tissue must be disaggregated before culture, and only a drop of medium is added on each piece so that the biopsy attaches on the surface and the cells start dividing.

Blood culture- Some cells, particularly lymphocytes do not undergo cell division and must be stimulated to divide by addition of a mitogen. The cell division is accelerated by adding PHA (phytohaemaglutinin). After setting-up of culture, once the requirements are met, the cells are allowed to grow under optimal conditions for an appropriate time. This time depends on the kind of cell involved. Subsequently, the dividing cells are collected at metaphase. A mitotic inhibitor is used to obtain adequate number of cells in metaphase. Colcemid binds to the protein tubulin and obstructs formation of spindle fibers preventing separation of

sister chromatids at the anaphase. However, in such mitosis the chromosomes are very short and condensed. Subsequently, cell synchronization methods were developed where cells are harvested at late prophase rather than metaphase. This is essential for high-resolution cytogenetics. It can be achieved by adding an excess of thymidine, methotrexate, 5-fluorodeoxyuridine (FdUrd), 5-bromo-2'-deoxyuridine (BrdUrd). These compounds inhibit DNA synthesis by disturbing the de novo synthesis of one pyrimidine base, thus blocking at the S-phase (Drouin et al., 1994). After synchronization, inhibition can be released by washing out the inhibitor. Hence, by performing each step at the right time, a good quantity of cells in late prophase to prometaphase can be harvested. After release of the cells they are given hypotonic shock to swell the cells. This is followed by fixation; the fixative must be freshly prepared to obtain good chromosome quality. Fixed cells from suspension are dropped onto clean slides under optimal temperature and humidity. Later the slides are aged overnight at 60°C for chromosome banding.

A large number of banding and staining techniques have been developed. On the basis of the unique banding pattern of each pair of chromosomes, they are identified and classified according to the nomenclature. This classification was set up in 1960, at the Denver Conference, by a group of cytogeneticists and has been upgraded since then in the following conferences of the ISCN (International System for Human Cytogenetic Nomenclature).

1.4.2) **Banding**- Differentially stained regions produced on a chromosome as a result of treatment with chemicals and dyes is referred to as a "band". Chromosome banding can

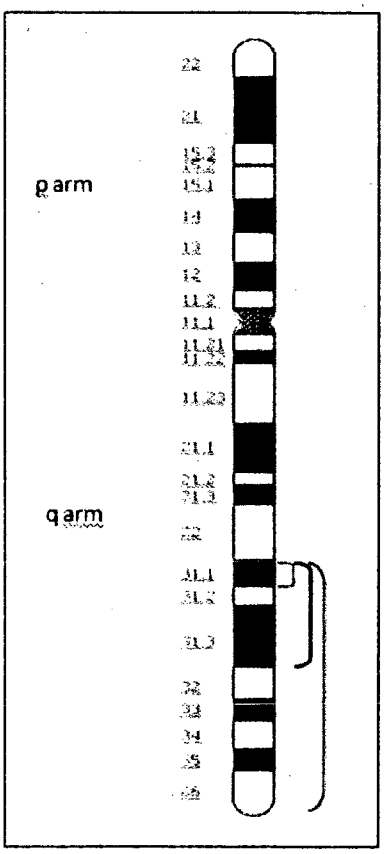
be morphologic (structural) and dynamic (replication) (Drouin et al., 1994). Morphological banding is the result of the chemical heterogeneity that exists along the length of the chromosome. Dynamic banding on the other hand relies on the incorporation of a base analog, usually BrdUrd, into DNA during the synthesis phase.

Another classification divides the banding techniques into two groups: (a) those that result in the distribution of bands on the entire length of chromosome, such a G, Q, and R-bands, and (b) those that stain specific chromosome structures. These methods reveal constitutive heterochromatin (highly compact, late-replicating, genetically inactive), like C-Bands (centromere), T-Bands (telomere) and NORs (nucleolus organizing regions).

1.4.2.1) Q-Banding- Q-banding was the first banding method developed for human chromosomes (Caspersson et al., 1968; Caspersson et al., 1970). Certain fluorochromes, such as quinacrine dihydrochloride, bind to DNA and produce banding patterns of bright and dull fluorescence when excited with proper wavelength of light. The A-T rich sites are brightly fluorescing regions.

1.4.2.2) G-Banding- Giemsa banding replaced Quinacrine banding in the 1970s. G-banding is the most widely used method in cytogenetic laboratories; GTG (G-bands by trypsin using Giemsa) is most common. Prepared and aged slides are treated with the enzyme trypsin and then stained with Giemsa producing light and dark bands ***(Figure 15)***. The G-bands that correspond to the bands darkly stained after GTG banding, are late replicating (Comings 1978), G-C poor (Cuny et al., 1981), heterochromatic regions of chromosomes, while the R-bands that correspond to the bands lightly stained after GTG banding are early replicating, G-C rich, euchromatic regions. Each chromosome after

staining consists of a continuous series of dark and light bands. Chromosomes are then arranged according to their banding pattern. The bands and regions are numbered from the centromere outward ***(Figure 15)***. A band is further subdivided in to **sub-bands** depending on the resolution. A **region** is defined as an area of chromosome lying between two adjacent **landmarks**. **Landmarks** are consistent and distinct morphological features important in identifying chromosomes. For example 7q31.1 means chromosome 7, long arm, region 3, band 1, sub-band 1 in that order. **Figure: 16a, b, c** and **d**, shows GTG performed on the three cases that were studied by us.



http://www.genet.sickkids.on.ca/chromosome7/ (modified)

Figure 15: *Idiogram of G-banding pattern for normal human chromosome 7 at approximately 550 band level. Indicated with orange is a* ***sub-band****, with blue is a* ***band*** *and with red is a* ***region****.*

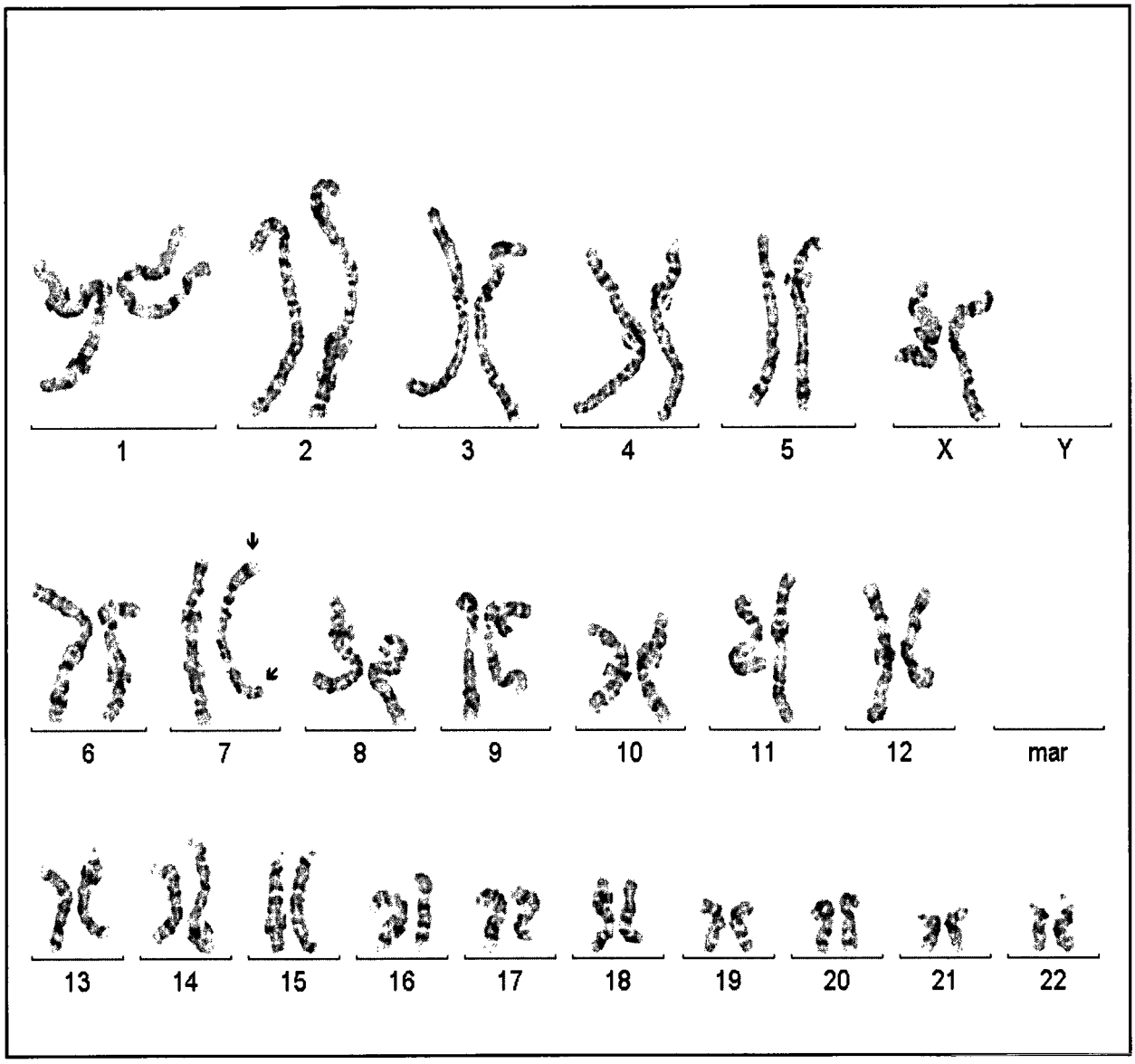


Figure 16a: *High-resolution karyotype of patient 1.*

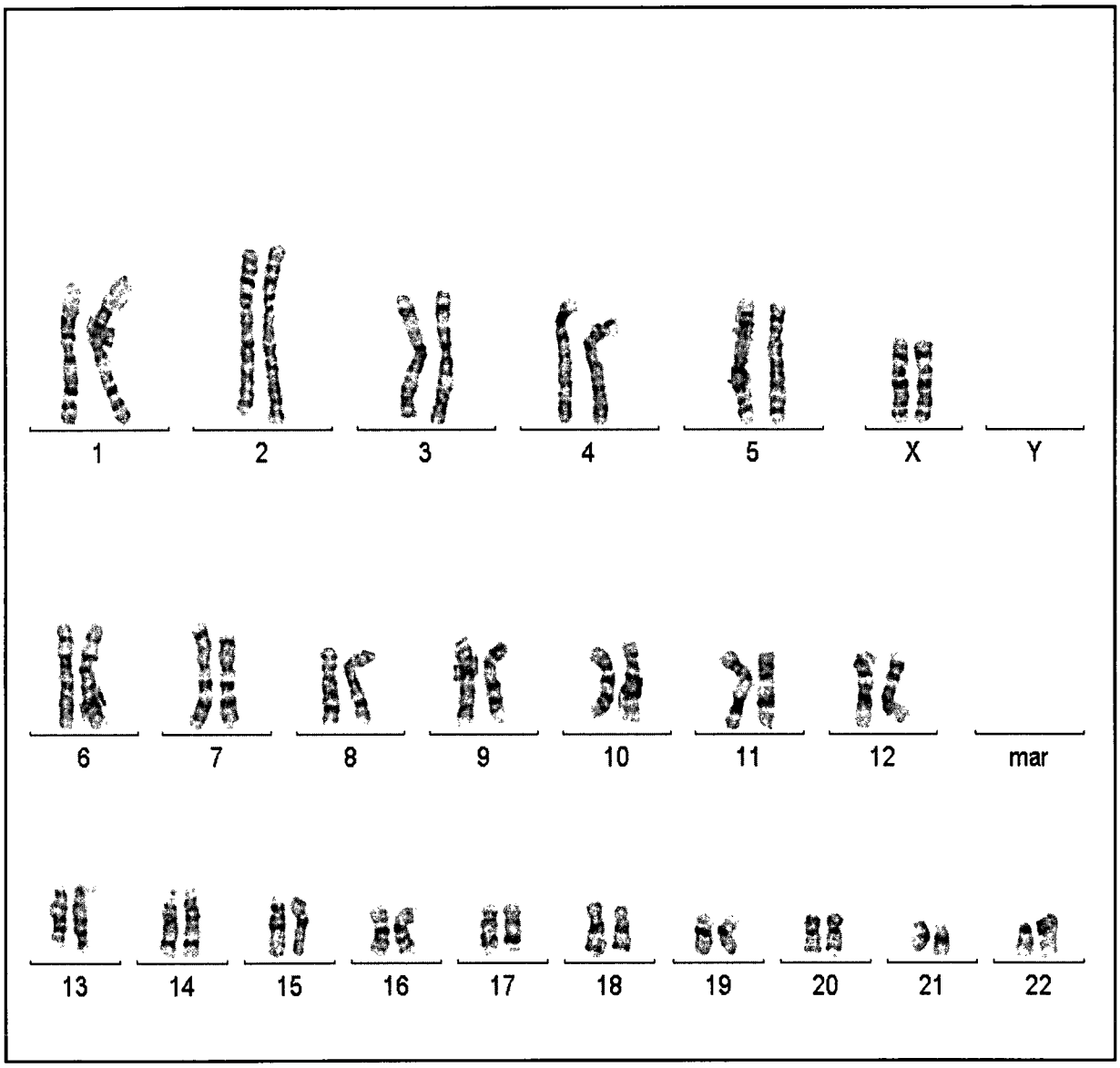


Figure 16b: *Low-resolution karyotype of patient 1.*

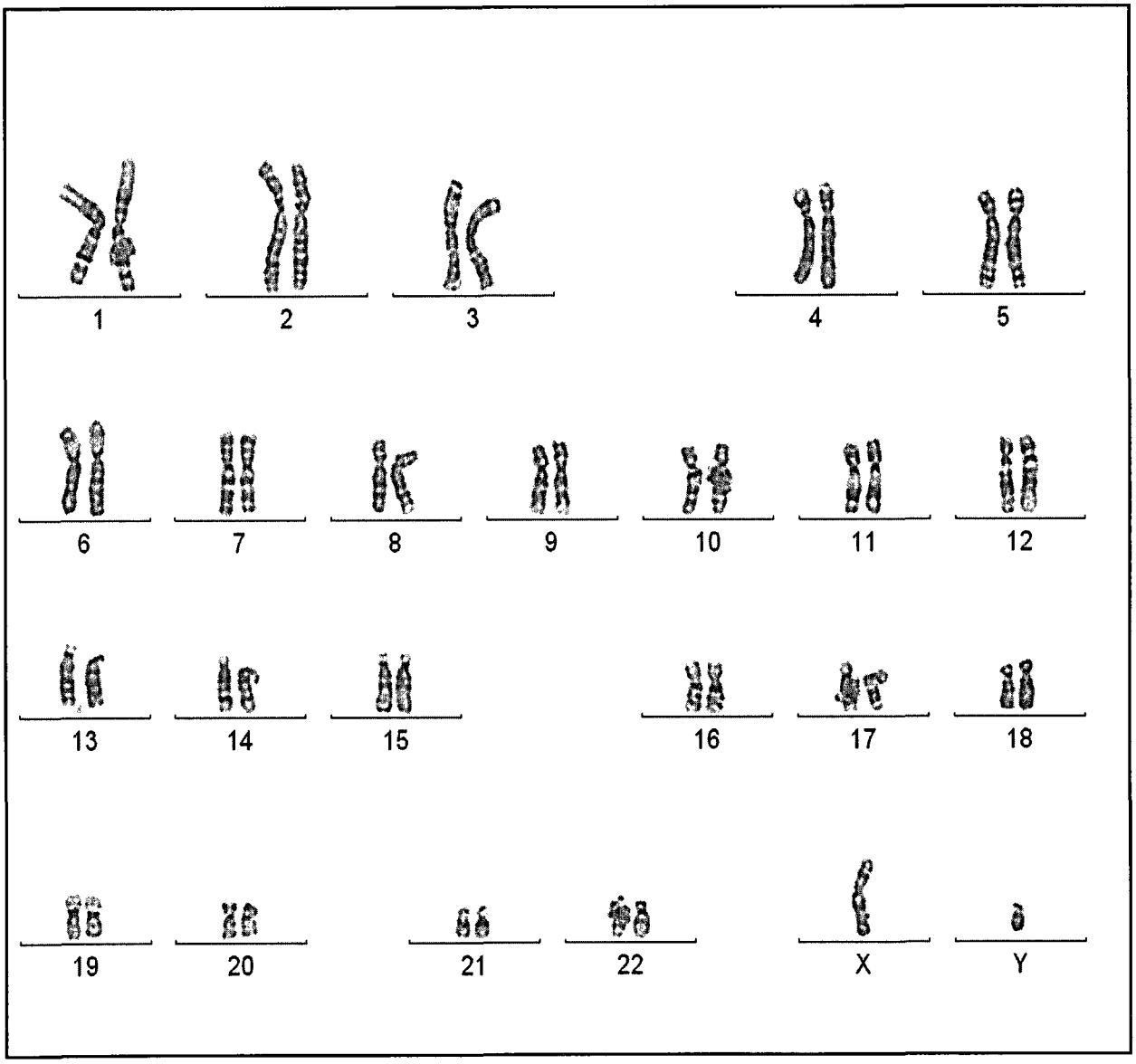


Figure 16c: *Low-resolution karyotype of patient 2.*

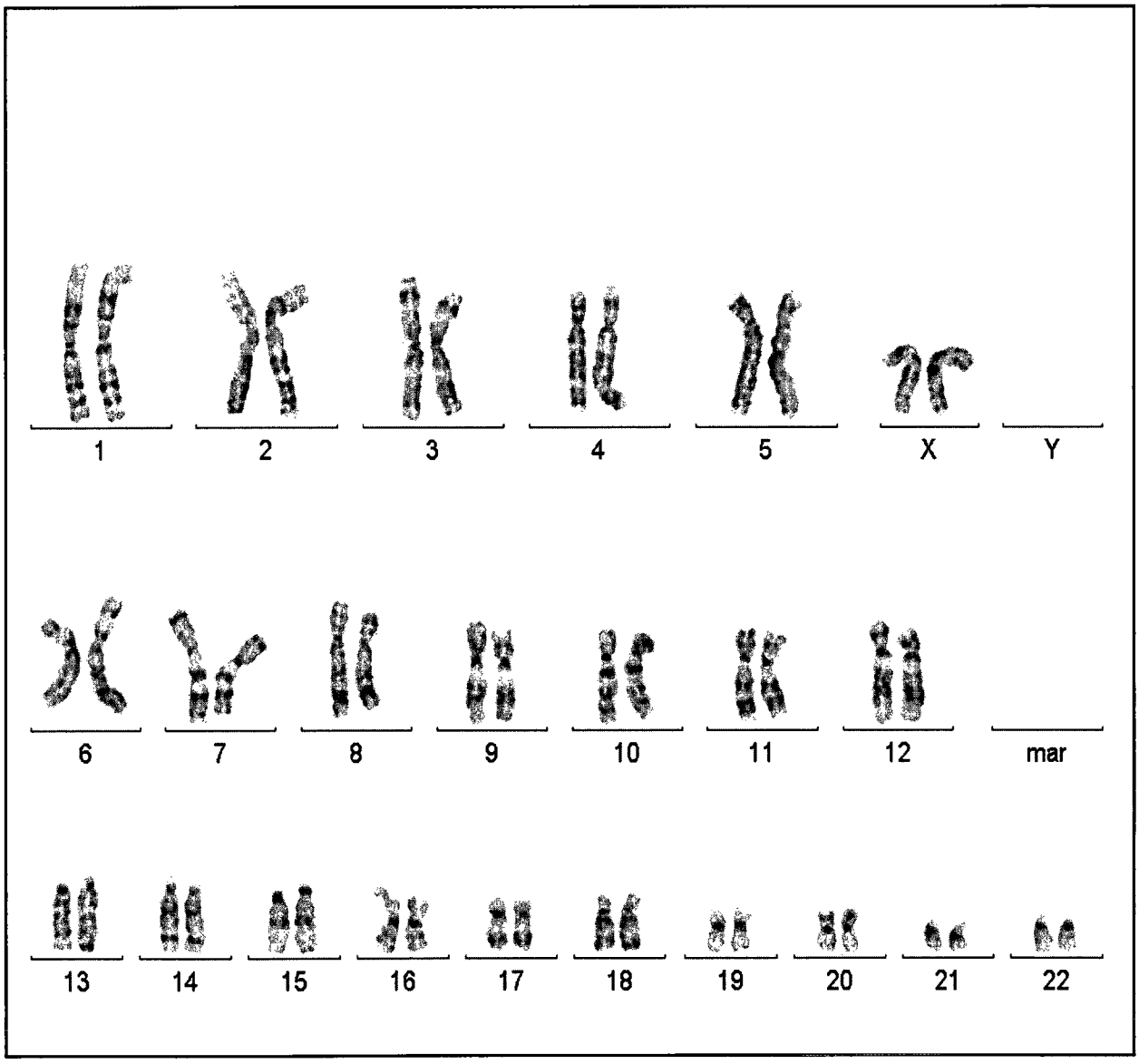


Figure 16d: *Low-resolution karyotype of patient 3.*

1.4.2.3) R-Banding- R-banding is the reverse of G-banding. It was discovered by Dutrillaux and Lejeune (Dutrillaux et al., 1971). R morphological bands can be obtained by thermal denaturation of chromosomes. Indeed, R-bands are less sensitive to heat and can be stained using Giemsa to be viewed (Richer et al., 1983). R-bands replicate early (Comings 1978) and are GC rich (Cuny et al., 1981).

1.4.2.4) C-Banding- C-banding techniques stain the constitutive heterochromatic regions around the centromeres. C-band regions contain highly repetitive, late replicating sequences of DNA.

1.4.2.5) T-Banding- T-banding involves staining only the telomeres of the chromosomes.

1.4.2.6) NOR Staining- This technique selectively stains the nucleolar organizer regions located on satellite stalks of acrocentric chromosomes. NOR staining is useful in identifying a marker chromosome or rearrangements involving acrocentric chromosomes.

Banding techniques are very efficient in detecting large rearrangements ($\geq$ 5Mb), but smaller chromosomal rearrangements often go unnoticed especially in regions where banding pattern is not distinctive. With the advent of molecular cytogenetic techniques it has become possible to obtain a higher resolution and to detect rearrangements of smaller magnitude.

1.5) Molecular cytogenetic techniques

ISH (In *Situ* Hybridization) - Joe Gall and Mary Lou Pardue in 1969 used DNA-RNA hybridization to localize genes that encode rRNA, this was the first *in situ* hybridization analysis reported (Gall et al., 1969). It was based on complementarity of bases, A with T and G with C. Radioisotope labeled probes were hybridized to cell preparations and detected using autoradiography. This was followed by the introduction of fluorescently labeled antibodies that recognized specific DNA-RNA hybrids (Rudkin et al., 1977).

1.5.1) FISH (Fluorescent *In Situ* Hybridization) - Later, chemical coupling of a fluorochrome to an RNA probe was used for direct visualization of the target (Bauman et al., 1980). Currently, in FISH technique, a fluorescently labelled DNA probe is hybridized to cytological targets such as metaphase chromosomes, interphase nuclei, fixed tissues, cell smears, or extended chromatin fibers (Fiber-FISH). Interphase FISH is advantageous in the rapid screening of many nuclei without the need for cell culture to get metaphases. FISH can be used both for unique and repetitive sequences. It is used to detect and localize the presence or absence of specific DNA sequences on chromosomes. On a broader scale, this technology can be utilized for the characterization of chromosomal rearrangements and marker chromosomes (Blennow et al., 1993; Pinkel et al., 1988), the detection of microdeletions (Ledbetter 1995) and the prenatal diagnosis of common aneuploidies (Klinger et al., 1992; Ward et al., 1993).

FISH is a technique that allows DNA sequences to be detected on metaphase chromosomes or on interphase nuclei. There are three major categories of DNA sequence used as probe for FISH studies.

1.5.1.1) Satellite sequence Probes – Satellite sequences are polymorphic, repetitive DNA sequences that are present in the genome but do not code for gene products. Different individuals have variations in the number of copies of these DNAs. Locations of such DNA are centromeres (alpha-satellite DNA), telomeres (TTAGGG repeats), tip of acrocentric chromosomes (β-satellite DNA); and classic satellite I DNA found on chromosomes 1, 9, 16, and Y. These probes are useful in determining the number of specific chromosomes present on interphase or metaphase.

1.5.1.2) Unique sequence Probes – These probes target sequence that are not repeated in the genome and may code for genes. They are aimed at unique sequences in subtelomeric regions or genes such as c-*myc*, *her-2-neu,* etc and can be used in the identification of microdeletions.

1.5.1.3) Whole chromosome Paints (WCP) – Whole chromosome probes are composed of numerous unique and repetitive sequences, each derived from one entire chromosome (Cremer et al., 1988) ***(Figure 17)***. These probes are designed to use on metaphase chromosomes only. WCPs are used to detect subtle or cryptic translocations, insertions or to determine the composition of marker chromosomes.

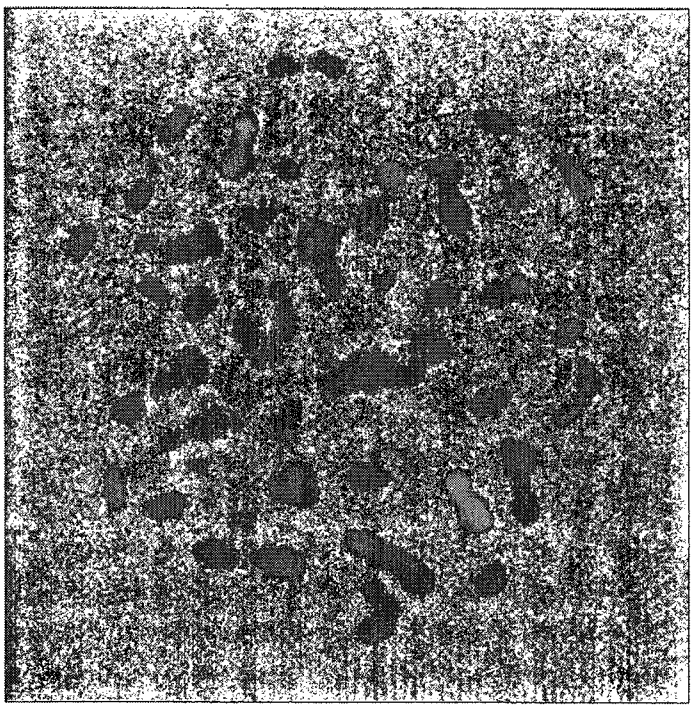


Figure 17: Whole chromosome painting on blood metaphase of case 1 on chromosome 7, confirming there are no rearrangements.

Other techniques

1.5.2) M-FISH- (Speicher et al., 1996) Using Multiplex-FISH (M-FISH), all the 24 chromosomes are painted with a different color using probes labeled with combinations of multiple fluorochromes. Images are collected with a fluorescence microscope that has filter sets for each fluorochrome. It is an approach that permits the simultaneous identification and analyses of all the chromosomes.

1.5.3) SKY- (Schrock et al., 1996) Spectral Karyotyping is an automated chromosome painting procedure. The principle is same as M-FISH except that an interferometer is used for fluorochrome discrimination and imaging.

1.5.4) Multicolor-Banding- (Chudoba et al., 1999) Multicolor-banding is based on differentially labeled overlapping micro dissection libraries. This allows the chromosome to have various colored bands.

1.5.5) Fiber-FISH- (Heng et al., 1992) DNA fibers are released from the interphase nucleus on microscope slide and hybridization is done. It offers a high-resolution approach for physical mapping and genome research.

1.5.6) Quantitative-FISH/PNA-FISH- This technique is used to measure telomere length. Telomeric PNA (peptide nucleic acid) probes are hybridized to normal metaphase spreads and the intensity of the signals is measured using software to assess in determining the relative length of the telomeres.

1.5.7) PRINS (Primed *in situ* Labeling) was designed by Koch *et al* (Koch et al., 1989). This method is based on annealing of specific short oligonucleotide primers to the denatured DNA, followed by subsequent primer extension using *taq* DNA polymerase and simultaneous labeling of the target sequences with a fluorochrome. However it is specific for repetitive sequences only. This technique has been used to detect aneuploidies efficiently.

1.6) Molecular genetics techniques

1.6.1) Microsatellite analysis – Parent of origin studies by genotyping can be performed using polymorphic markers. The higher the heterozygosity, the more informative the marker is i.e. if the parents carry different alleles, the origin of each allele in the offspring can be determined accurately. Microsatellites are short tandem repeat (STR) markers which are polymorphic DNA loci present throughout the genome and contain a repeated nucleotide sequence. These loci are amplified using primers by PCR and different sized alleles are separated using polyacrylamide gel electrophoresis. Microsatellite markers can be used to search for irregular allele inheritance, detection of deletions, or uniparental disomies (Ghaffari et al., 1998).

1.6.2) Comparative Genomic Hybridization (CGH) – Comparative Genomic Hybridization was first developed in 1992 (Kallioniemi et al., 1992), to map genomic imbalances in solid tumors since preparation of high-quality metaphase spreads is difficult in solid tumors. Further, du Manoir et al. (du Manoir et al., 1993) used it to detect complete or partial chromosome gains and losses. It is now extensively used in clinical and research labs. In this technique, DNA is extracted from the test sample and a normal reference sample. It is then differentially labeled; traditionally test sample is labeled in green and normal reference in red. The combined probes are then co-hybridized to normal target metaphase chromosomes. If a region is amplified

in the test sample the corresponding region on the metaphase chromosome becomes green while if a region is deleted it becomes red. The red/green ratio is then quantified using digital image analysis. The metaphase chromosomes are replaced by mapped clones in case of array-CGH (aCGH) that offers a higher resolution screening (Pinkel et al., 1998; Solinas-Toldo et al., 1997). The highest resolution for aCGH is now provided by oligonucleotide arrays (Carvalho et al., 2004; Lucito et al., 2003; Zhao et al., 2004).

One of the main advantages of CGH is that it does not require any prior knowledge of the chromosome imbalance involved.

2. Terminal Deletion of Chromosome

Exposure to ionizing radiation or other exogenous or endogenous chromosome-breaking agents during pregnancy can produce double-strand breaks in DNA resulting in chromosomal deletion. These can range in size from a few base pairs to many megabases in length.

A chromosomal deletion is usually a sporadic event that occurs in the egg or sperm before or after fertilization. The sperm and egg contain 23 chromosomes each. After fertilization the chromosome number restores to normal 46 in the zygote, each parent contributing one chromosome each (23 in total) out of the homologous pair of every chromosome. A deletion may thus cut out important genes, which often results in an abnormal genetic condition. Deletions that are not inherited from the parents and occur *de novo* are called as sporadic deletions. In other cases it can be inherited, where the parent is a carrier of a chromosomal anomaly like a deletion or a translocation. Balanced chromosomal rearrangements can generate gametes with genetic imbalance (partial monosomy/trisomy) due to aberrant segregation during meiosis. Various chromosomal deletions have been reported till date, some of them resulting in deletion syndrome, each syndrome characterized by a variety of symptoms. Below are described one common and one rare terminal deletion, followed by the 7q terminal deletion, to compare and understand the etiology of terminal deletion syndromes.

2.1 Terminal Deletion Syndromes

2.1.1 Monosomy 1p36:

Deletion of chromosome band 1p36 or monosomy 1p36 has been considered to be one of the most common chromosome deletion syndromes, with an estimated incidence of 1 in 5,000 to 1 in 10,000 live births (Heilstedt et al., 2003). This syndrome was first reported in 1980 and occurs due to the partial loss of material from the short arm of chromosome 1. The breakpoints range from bands 1p36.13 to 1p36.33 in this cytogenetic syndrome. The majority of cases reported in the literature are with pure terminal deletions. The clinical symptoms include-developmental delay, mental retardation, brain abnormalities, heart abnormalities, characteristic craniofacial appearance, deep-set eyes, midface hypoplasia, broad nasal root/bridge, long philtrum, and pointed chin. This syndrome is responsible for ~1% of all cases labeled as "idiopathic mental retardation" (Giraudeau et al., 2001). Other features observed were microbrachycephaly, epicanthal folds, posteriorly rotated malformed ears, seizures, hearing impairment, microcephaly etc (Battaglia et al., 2008). Individuals with 1p36 syndrome often face serious physical disabilities. This syndrome includes a wide range of symptoms variable in nature.

2.1.2 14q terminal deletion:

Terminal deletions of chromosome 14 (14q32) are quite rare. This deletion leads to 14q terminal deletion syndrome. About 20 such patients have been described in the literature. The most common clinical manifestations are- microcephaly, broad nasal bridge, high forehead with lateral hypertrichosis, long and broad philtrum, high arched palate, epicanthic folds, single palmar crease, hypotonia, mental retardation and developmental delay (Schlade-Bartusiak et al., 2009). This syndrome in not generally associated with multiple congenital anomalies. The symptoms in these patients are sufficiently specific to allow delineation of a clinically recognizable syndrome (van Karnebeek et al., 2002).

2.1.3 7q terminal deletion:

More than 100 cases of 7q terminal deletion have been described in the literature. The first such case was described by De Grouchy and colleagues (De Grouchy et al., 1968), which was initially thought to be a case of 6p deletion instead. The term "7q deletion syndrome" was coined by Harris and colleagues (Harris et al., 1977) when they described four cases carrying terminal deletion 7q32 with the following clinical manifestations – growth deficiency, developmental delay, prominent forehead, bulbous nasal tip, abnormally shaped ears, cleft lip and palate, genital abnormalities and simian creases. Since then, many other clinical features have

been associated with 7q terminal deletion patients, such as holoprosencephaly (HPE), sacral agenesis, mental retardation, feeding difficulties and hearing problems. Some of the symptoms described above are common to other chromosomal imbalances while others are specific to 7q deletion. A review of the cases described before reveals that the patients carrying an apparently similar deletion, for example 7q36, present variable phenotypes. This tendency was also observed in the three patients we studied. Also, the severity of holoprosencephaly, a common developmental defect of the forebrain, varies widely in different carriers (Roessler et al., 1996). Thus, it becomes essential to characterize the deletion, to locate the exact breakpoint, to study the genes involved and to evaluate the cause of such phenotypic variability. Possibility of role of modifier genes, polymorphism and/or position effect is discussed.

Interest of our study

Chromosomal anomalies contribute to a significant number of miscarriages or induced abortions. Those anomalies that are compatible with life usually result in mental retardation, growth retardation and other malformations in the fetus/infant. Consequently, it is important to investigate chromosomal aberrations that are compatible with post-natal life but intervene with the normal development of the embryo. This gives a better understanding of the anomaly so that a better diagnosis and care can be provided. It is further interesting to analyze why certain anomalies present heterogeneous phenotypes. We studied three patients who carried 7q terminal deletion, all with variable symptoms. With the recent advancement in cytogenetic techniques it has become possible to characterize such deletions at a very high-resolution. Using these tools we localized the deletions, stratified the symptoms associated with 7q35 and 7q36 terminal deletions, established a genotype-phenotype correlation along with the literature review of similar cases.

Objectives

- Collect the clinical information of the three patients.

- Identify the chromosomal anomaly in these patients using conventional cytogenetic techniques, such as low- and high-resolution karyotype, followed by performing the same techniques in the parents to determine whether the anomaly has been transmitted or has occurred *de novo*.

- Establish whether the deletion is interstitial or terminal using subtelomeric FISH on chromosome 7.

- Search for mosaicism (case 1), by performing subtelomeric FISH on chromosome 7 on cells from skin (fibroblasts) and buccal smear (epithelial cells).

- Determine whether the chromosome that carries the deletion is maternally or paternally derived using microsatellite analysis and locate the exact breakpoint.

- To look for cryptic aberrations using very high-resolution array-CGH, determine the precise length of the deleted segment and the genes lost.

- Confirm the result of array-CGH (Xq28 duplication) for case 3, using subtelomeric and centromeric FISH on chromosome 7 and X.

- Review the literature with cases carrying 7q terminal deletion and other chromosomal terminal deletions and observe the clinical symptoms that these cases manifested.

- Establish a genotype-phenotype correlation based upon the published data and our three cases and stratify the symptoms associated with these deletions.

- Finally, speculate why patients with similar 7q terminal deletion present variable phenotypes.

Résumé (de l'article)

Introduction- La monosomie partielle du bras long du chromosome 7 est associée à un large spectre de manifestations cliniques, notamment l'holoprosencéphalie et l'absence de sacrum. Cependant, il reste à définir une relation claire entre le génotype et le phénotype dans les différents cas du syndrome de délétion 7q.

Objectifs- Caractériser trois cas cliniques possédant tous une délétion du bras long du chromosome 7 mais présentant des phénotypes cliniques différents.

Matériels et méthodes- Les caryotypes des trois patients ont été réalisés en utilisant la méthode de marquage GTG de base ainsi qu'à haute résolution. L'étude par hybridation *in situ* en fluorescence de sondes subtélomériques et l'analyse des microsatellites à l'aide de différents marqueurs spécifiques du chromosome 7 ont été effectuées. Nous avons également réalisé une exploration par micropuce sur ces trois cas.

Résultats- Le 1er patient portait une délétion *de novo* de 6.6 Mb au niveau de la bande 7q36.2 du chromosome 7 hérité de la mère. Le point de cassure de cette délétion était située entre les marqueurs D7S483 et D7S798. Cet enfant a comme unique anomalie phénotypique une agénésie partielle de sacrum. Le 2^{e} cas était porteur d'une délétion terminale *de novo* de 13.8 Mb localisée à la bande 7q35 du chromosome 7 hérité du père. Le point de cassure de cette délétion était localisée entre les marqueurs D7S661 et D7S2426. À l'autopsie, ce fœtus présentait une pyélectasie du rein droit avec une

hypertrophie pelvienne. Le 3^{e} patient est porteur d'une délétion terminale *de novo* de 7.2 Mb au niveau de la bande 7q36.1 et d'une duplication de 1.2 Mb localisée à la bande Xq28. À l'exception d'une microcéphalie notée à la naissance, ce dernier cas n'avait aucun signe phénotypique anormal. Le point de cassure de cette délétion était localisée entre D7S642 et D7S483. Il est à noter que ces deux derniers cas ne présentaient ni HPE, ni agénésie du sacrum.

Conclusion- Les cas présentés ici soulignent l'importance et le rôle des facteurs environnementaux, des gènes modificateurs et/ou de l'effet de position sur les différents phénotypes observés dans le cas de délétion 7q.

Three New Cases of Terminal Deletion of the Long Arm of Chromosome 7 and Literature Review to Correlate Genotype and Phenotype Manifestations

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Key words: 7q deletion, chromosome 7, holoprosencephaly, sacral agenesis, array-CGH

Short title: Terminal deletion of the long arm of chromosome 7.

ABSTRACT

Introduction- Partial monosomy of the long arm of chromosome 7 (7q) has been characterized by wide phenotypic manifestations, but holoprosencephaly (HPE) and sacral agenesis have always been associated with this chromosomal deletion. A clear relationship between genotype and phenotype remains to be defined in the 7q deletion syndrome.

Objective- To investigate three patients with a 7q terminal deletion presenting variable phenotypes and compare with similar deletion cases in the literature to stratify the phenotypes associated with 7q35 and 7q36 terminal deletion patients in context of 7q deletion syndrome.

Materials and Methods- G-banding was carried out at low and high-resolution. Fluorescent *In Situ* Hybridization (FISH) using sub-telomeric probes was performed followed by microsatellite analysis using markers specific for chromosome 7. Finally, a whole genome microarray analysis was also performed.

Results- Patient 1 was carrying a *de novo* terminal deletion at band 7q36.2 on the maternally derived chromosome, located between the markers D7S483 and D7S798. Patient 2 was carrying a *de novo* terminal deletion at band 7q35 on the paternally derived chromosome, located between the markers D7S661 and D7S2426. Patient 3 had a *de novo* terminal deletion at band 7q36.1 on the paternally derived chromosome, located between the markers D7S642 and D7S483. A small Xq28 duplication was also identified by array-CGH, which was missed by a standard karyotype. Patient 1 presented partial sacral agenesis. However neither HPE nor sacral malformations were observed in patients 2 & 3.

Conclusion- Our patients with terminal 7q deletion presented heterogeneous phenotypic manifestations which highlights the significance and role of environmental factors, modifier genes and/or polymorphism. Growth retardation was the most frequent symptom found in both

7q35 and 7q36 patients. The presence of HPE and sacral malformation together was seen in less than 10% of the cases in both kinds of deletion. HPE was mostly related to the cases with an unbalanced translocation.

INTRODUCTION

7q deletion is a rare chromosomal structural aberration, which involves the loss of the terminal region of the long arm of chromosome 7. The first case of a 7q deletion was described in 1968 (De Grouchy et al., 1968). The carriers of this deletion present a wide variety of symptoms while sharing a few common features. The main clinical features associated with it are – growth and motor retardation, mental retardation, hypotelorism, hypotonia, microcephaly, upslanting palpebral fissures, prominent forehead, epicanthal folds, cleft lip, flat and broad nasal bridge, bulbous nasal tip, micrognathia, abnormal palmar crease, feeding problems, and low set ears among others, described as 7q deletion syndrome in 1977 (Harris et al., 1977) and reviewed in 2005 (Lukusa et al., 2005). However, despite several decades of research trying to decipher the molecular mechanisms of the symptoms in 7q deletion syndrome, until now a clear relationship between genotype and phenotype remains to be defined.

Furthermore, 7q terminal deletion has been time and again linked with sacral agenesis (Nowaczyk et al., 2000; Wang et al., 1999) and midline embryonic field defects like holoprosencephaly (HPE), which can range from cyclopia to a single central upper inscisor (Roessler et al., 1996). HPE is generated by common and complex malformations affecting the cleavage of the developing forebrain and is accompanied with defects in the mid face (Benzacken et al., 1997). It is divided in three types: alobar, semilobar and lobar HPE and occurs

in 1/16,000 live births and in 1/250 spontaneous abortions (Kanafani et al., 2007. The gene SHH present on 7q36 on loci HPE3 was identified to be one of the genes to cause HPE {Belloni, 1996 #2; Roessler et al., 1997a; Schell-Apacik et al., 2003). Genetically, chromosomal aberrations such as triploidies, trisomies 13 or 18, mendelian inheritance or rearrangements of at least those 12 gene loci located on 11 different chromosomes are implicated in the pathogenesis of HPE (Kanafani et al., 2007), (Roessler et al., 1998). Also, this clinical feature is frequently associated with sacral agenesis in 7q36 deletion. Sacral agenesis also ranges from partial agenesis of the coccyx to complete absence of the sacral, lumbar and seldom thoracic vertebrae (Pang 1993). Currarino syndrome occurrence is 1.3/10,000 live born and it consist of the triad of anorectal malformation, a sacrococcygeal defect, and a presacral mass, recognized as a syndrome in 1981 (Currarino et al., 1981). In addition, *HLXB9* was identified as the major gene causing sacral agenesis also residing on 7q36 (Ross et al., 1998). *HLXB9* misense mutations and deletions have been identified in some Currarino's syndrome (Belloni et al., 2000).

However, deletion of these genes does not necessarily imply a severe phenotype. The severity of symptoms varies between individuals carrying an apparently cytogenetically similar deletion (Frints et al., 1998; Lukusa et al., 2005). Most of the patients described before have been evaluated using standard cytogenetic techniques (G or R banding), which gives a relatively low-resolution localization of the breakpoint limited to the band level. Here we report a detailed cytogenetic and molecular investigation of three patients carrying a 7q deletion. We performed microsatellite analysis and array-CGH to precisely define the breakpoint, to study the genes lost and to investigate the symptoms usually associated with 7q deletion syndrome. We stratified the

symptoms manifested by 7q35 and 7q36 deletion patients and investigated the most common phenotypes and their percentage (based on a total of 55 cases).

CLINICAL REPORTS

Patient 1

The girl was born to 23-year old parents and is now 9 years old. At birth her height was 45 cm, weight was 2.5 kg and a cranial perimeter of 30.5 cm was observed. She walked at 16 months. A neurologic examination revealed axial hypotonia. She spoke at three and a half years and also suffered moderate hearing problems. The following clinical symptoms were observed: microcephaly, psychomotor retardation, webbed shortened neck, multiple nevi on different areas of the body, marble-like pattern on the skin of the whole body similar to fishnet-like blotches, small bilateral cubitus valgus, broad chest with shield-like thorax, widely spaced nipples, congenital partial sacral agenesis and skeletal deformity inducing to a lumbar congenital hyperlordosis. There were slight cranio-facial dysmorphism signs such as oval face shape, large forehead, small eyes, bilateral and symmetric well-shaped ears normally implanted, eumorphic macrotia with salient antihelix and big antitragus. At the exam of the oral cavity and palate, we noted thin lips, hypoplastic orbicularis oris muscle leading to undergrowing median area of lower lip, malocclusion of teeth, slightly large palate, but globally, the central segment of the face, comprising the forehead, supraorbital ridges, nose, philtrum, and primary palate, which derived from the frontonasal process, was close to normal; buccal cavity was otherwise normal. She is at the Tanner stage II characterized by isolated premature thelarche II (breast buds are formed and palpable, with small area of surrounding glandular tissue; areola begins to enlarge), pubarche I (no pubic hair at all) corresponding to the prepubertal stage, no menarche and no premature

adrenarche. MRI of the brain revealed complete corpus callosum. The mother suffered from pyelonephritis and was on antibiotics for the same during 12-13 weeks of the pregnancy. The father had hearing problems in one ear due to fused bones.

Patient 2

An amniocentesis has been performed for high risk of trisomy 21 at the prenatal serum marker screening test. A 7q deletion was detected and the parents decided to interrupt the pregnancy. This is a male fetus that was aborted at 22 weeks. The mother was 32 years old and the pregnancy was normal. Fetal ultrasound showed an occipitofrontal circumference at the 5th percentile. An autopsy determined the weight to be 390.6 g, height 25 cm, head circumference 18 cm (5th percentile), and circumference of thorax 16.5 cm. There was no facial dysmorphism, nasal and buccal cavities were well configured. There was a right kidney pyelectasia with no kidney dysplasia. Thorax, abdomen and back were without any particularity. The respiratory, cardiovascular, gastrointestinal, endocrine and hemolymphatic system were normal. There were no signs of HPE on examination of the nervous system. The cerebral hemispheres were symmetric and the ventricles were not dilated.

Patient 3

This is a 2 years and 8 months old girl. At the time of examination, her weight was 10.2 kg, height was 84 cm and cranial perimeter was 43 cm, all below the 3rd percentile. She had microcephaly since birth. Development was acceptable in the first six months but slowed down later in life. She never developed any language and has difficulties walking. Hearing and vision

were normal. MRI of the brain was found to be normal. Dentition is normal. Thus, she had no signs of HPE. An X-Ray of the pelvic region ruled out any sacral malformations.

MATERIALS AND METHODS

Conventional Cytogenetics

Sampling

After genetic counseling, 5 to 10 ml of peripheral venous blood was collected in a vacutainer sodium heparin tube (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) from the proband, mother and father. Skin biopsy and buccal smear from the patient 1 was taken and suspended in complete medium (RPM1, DMEM, 10% FBS, 2% antibiotics) and HBSS (Hank's balanced salt solution) respectively. Approximately 20 ml of amniotic fluid was obtained through the usual procedure from patient 2.

Low resolution karyotype

Blood (0.5 ml) was taken in Falcon polypropylene tubes (15 ml) for each sample with 10 ml of culture medium (88% RPM1, 10% FBS, 1% glutamine, 1% PHA, 0.01% antibiotics). The blood culture was incubated at 37°C, 5% CO_2 (MCO-20 AIC, Sanyo electric Biomedical Co., Ltd, Japan). After 3 days, 50 µl of colcemide (10 µg/ml) was added for 1 h before harvest. For harvesting the tubes were centrifuged at 1500 rpm (*c.* 500 g, ICE centrifuge, HNSII model A Division of Damon, USA) for 7 min and the platelet-rich supernatant was discarded. The cells

were resuspended in a hypotonic solution (0.56% KCl) and incubated at 37°C for 10–15 min. After cytocentrifugation for 5 min at 2000 rpm (*c.* 667 g), the cells were fixed several times with freshly prepared precooled Carnoy's fixative (3: 1, methanol: glacial acetic acid). The pellet was resuspended in 1 ml of fresh Carnoy. We spread 15 µl of fixed cell suspension onto cleaned slides in a modified Thermotron environmental control unit (CDS-5, Thermotron, Amsterdam, Holland) at an optimal temperature (22°C) and humidity (55%). The slides were aged for a few days and GTG (G-bands by trypsin using Giemsa) was performed according to the standard protocol (Drouin et al., 1988).

High-resolution karyotype

Blood was cultured as described above, but after 3 days in incubation, the cells were blocked with thymidine (300 µg/ml final) and were incubated again. After 17 h, tubes were centrifuged at 1700 rpm for 7 min. The pellet was washed with HBSS. Cells were centrifuged again and 10 ml of release medium was added followed by 0.5 ml of BrdU (5-bromo-2'-deoxyuridine) to a final concentration of 30 µg/ml. Incubation was done for 4 h 45 min. Colcemide was added 50 µl in each tube as above. This was incubated at 37°C in water bath for 15 min. Rest of the protocol was same for harvest and G-banding (Drouin *et al.* 1988).

Fifteen metaphases were counted and 5 metaphases were karyotyped.

Amniotic fluid culture

Amniotic culture from patient 2 was done following the standard protocol (Barch et al., 1997).

Fibroblast culture

Skin biopsy was cultured in the medium according to standard method to obtain fibroblast and fibroblastic metaphases. After the cells became confluent, they were harvested using standard protocol (Barch et al., 1997).

Buccal smear

Buccal smear was harvested using the same protocol as blood. The hypotonic shock was given in sodium citrate (0.4 %) for 20 min.

Molecular cytogenetics

Harvested sample was spread on clean slides under optimal conditions and incubated at 37°C overnight. FISH using sub-telomere probes for chromosome 7 in all patients was performed (Abbott Molecular Inc., Downers Grove, IL, USA). FISH was done on chromosome X in patient 3 after array results. We also performed Whole chromosome Painting (WCP 7, Abbott Molecular Inc., Downers Grove, IL, USA) on chromosome 7 in patient 1. Sub-telomere FISH on fibroblasts and buccal smear cells was done using same probes on chromosome 7. Before FISH, pre-treatment was done on buccal smear slides in 2xSSC for 30 min, digestion solution (50 ml water, 500 µl 1N HCl, 2.5 µl 10% pepsin) for 12 min at 37°C, 1X PBS 5 min, formaline (1 ml formaldehyde 37%, 800 µl $Mgcl_2$ 2.5M) 5 min, 1X PBS 5 min, followed by dehydration in 70%, 80% and 100% ethanol for 2 min each.

All slides were examined under Olympus BX61 microscopes equipped with appropriate filters at 1000X magnification. Imaging of selected cells was done using JAI M300 video CCD camera. The pictures were analyzed using the *in situ* imaging system (ISIS) software version 5.2 (MetaSystems Inc, Belmont, MA).

Fifty metaphases were examined for buccal smear and fibroblasts. Ten metaphases were counted for sub-telomeric FISH.

Molecular Genetics

Microsatellite analysis

To determine the exact breakpoint on a physical map and the parental origin of the deleted chromosome, microsatellite analyses were carried out with many flanking oligodeoxyribonucleotides as primers that map the chromosome 7. The DNA from mother, father and proband was extracted using QIAGEN Kit (QIAGEN Sciences, Maryland 20874, USA) and was typed with a series of polymorphic (CA)n microsatellite repeats. The microsatellite markers tested for the 3 patients are shown in table 1. The PCR was performed using 10 to 30 ng of genomic DNA, 2.5 mM of dNTPs (Roche), 1 μM of the forward and reverse primers. The forward primer was labeled with IRD700 (M13IRD700) (Li-Cor, Lincoln, NE, USA) for direct detection. The amplification reaction (Biometra, Montreal Biotech Inc thermocycler, Kirkland, Quebec, Canada) contained Hot Start Taq (5 U/μl) (Qiagen) was cycled for 35 cycles after preheating at 95°C for 15 min: 1) denaturation at 95°C for 40 s; 2) annealing at 55°C for 30 s; 3) extension at 72°C for 40 s. The amplified products were resolved on 6% polyacrylamide gel

using the Li-Cor Sequencer (NEN 4300L DNA analyzer, LI-COR Inc. Lincoln, NE) according to the manufacturer's protocol.

Array CGH

DNA from the mother, father and patient was extracted as mentioned above and array CGH was performed by Gene Dx (Perry Parkway, Gaithersburg, MD) using GenomeDx microarray, v3.0. The patient's DNA was labeled with red fluorescent dye and control DNA with green fluorescent dye. This was combined in equal ratio and hybridized to a glass slide coated with probes for whole genome. The array contained approximately 105,000 oligonucleotide probes spaced at an average distance of 37 kb (http://genome.ucsc.edu/cgi-bin/hgGateway). After hybridization the ratio of red to green was determined using analysis instrument.

RESULTS

Conventional cytogenetics

Low-resolution karyotype- In patient 1, both chromosomes appeared normal. A terminal deletion on the long arm of one of the chromosomes 7 was observed in patients 2 and 3 (Figure $1a_1$, 1b and 1c). The karyotype of the parents was normal in all the three patients.

High-resolution karyotype- Terminal deletion on the long arm of chromosome 7 was observed (patient 1) (Figure $1a_2$).

Patient 1- 46,XX,del(7)(q36)

Patient 2- 46,XY,del(7)(q3?5)

Patient 3- 46,XX,del(7)(q3?5)

Molecular cytogenetics

Only one red signal at 7p confirmed 7q terminal deletion in patient 1 (2a), patient 2 (2b) and patient 3 (2c). In patient 1, whole chromosome painting confirmed that there were no re-arrangements. Sub-telomeric FISH on buccal smear and fibroblasts confirmed that there was no mosaicism (Figure 18). An Xq duplication was later confirmed in patient 3 after array analysis (2d).

Patient 1- ish del(7)(qter-)(VIJyRM2000-)
Patient 2- ish del(7)(qter-)(VIJyRM2000-)
Patient 3- ish del(7)(qter-)(VIJyRM2000-) and ish der(7)t(X;7)(qter+,qter-) after array-CGH

Microsatellite analysis

Patient 1- The deletion was located between the markers D7S483 and D7S798 on maternally derived chromosome between bands 7q36.1 and 7q36.2. Shown in figure 3 is a deleted and a normal marker (Figure 3a and 3d).

Patient 2- The deletion was located between the markers D7S661 and D7S2426 on paternally derived chromosome between bands 7q35 and 7q36.1. Shown in figure 3 is a deleted and a normal marker (Figure 3b and 3e).

Patient 3- The deletion lies between the markers D7S642 and D7S483 at band 7q36.1 on paternally derived chromosome. Shown in figure 3 is a deleted and a normal marker (Figure 3c and 3f).

Array CGH

Patient 1- The deletion was located at chromosome region 7q36.2q36.3 from genomic position 152,213,519 to 158,818,165 bp. The estimated size of this genomic imbalance is 6.6 Mb. (Figure 4a). This deletion results in the loss of one copy of approximately 30 genes, which include OMIM genes SHH (OMIM#600725), HLXB9 (OMIM#142994), LMBR1 (OMIM#605522), and EN2 (OMIM#131310) (from UCSC OMIM gene track).

Patient 2- The deletion was located at chromosome region 7q35q36.3 from genomic position 144,999,574 to 158,818,166 bp. The estimated size of this genomic imbalance is 13.8 Mb. (Figure 4b). This deletion results in the loss of one copy of approximately 65 genes, which include OMIM genes SHH (OMIM#600725), HLXB9 (OMIM#142994), LMBR1 (OMIM#605522), and EN2 (OMIM#131310).

Patient 3- The deletion was located at chromosome region 7q36.1q36.3 from genomic position 151,623,385 to 158,818,166 bp. The estimated size of this genomic imbalance is 7.2 Mb. (Figure 4c). This deletion results in the loss of one copy of approximately 35 genes, which include OMIM genes SHH (OMIM#600725), HLXB9 (OMIM#142994), LMBR1 (OMIM#605522), and EN2 (OMIM#131310). In addition, a 1.2 Mb duplication was present at chromosome region Xq28q28 from genomic position 153,665,271 to 154,883,307 containing approximately 11 genes (Figure 4d).

Parental samples yielded normal results.

The cytogenetic formulas for the 3 patients are-

Patient 1- 46,XX,del(7)(q36.2)

Patient 2- 46,XY,del(7)(q35)

Patient 3- 46,XX,der(7)t(X;7)(q28;q36.1)

DISCUSSION

Several patients with 7q deletion have been described in the medical literature without characterizing the "exact" breakpoint and the role of the genes lost in the occurrence of the symptoms. This limitation has been mostly related to the low-resolution of cytogenetic tools used. We performed microsatellite analysis and array-CGH in order to overcome this limitation. The resolution limit has increased from 2-5 Mb to 0.3-0.5 Mb with the advent of these techniques.

The deletion 7q36.2, which we present in patient 1 is very small and rare, since most of the deletions that have been characterized until now were located either at 7q32, 7q35 or 7q36.1. To our knowledge this is the first case reported with 7q36.2 deletion, probably due to the limited cytogenetic tools available in the past. This patient (patient 1) presented with partial sacral agenesis that could be explained due to loss of the gene *HLXB9* present on band 7q36.3. According to the microsatellite and microarray results the gene *SHH* is also deleted in this patient. However she did not show any severe forms of HPE, but only had microcephaly which is considered to be the mildest expression of HPE by some authors. To explain her phenotype, we determined whether she carried mosaicism by doing sub-telomere FISH specific for chromosome 7 on fibroblasts and buccal smear cells. All tissue cells examined had a del(7qter) . The results reduced the possibility of the patient carrying two kinds of cell population, one normal and one abnormal that could have explained her phenotype.

The deletion in patient 2 (fetus) included the *SHH* and *HLXB9* genes, however, he neither presented HPE nor sacral dysgenesis. Even though it was a 22 weeks old fetus that was

terminated, it could be assumed that no other symptoms would develop later in the following weeks. Nevertheless regarding the literature with fetuses carrying a 7q terminal deletion reveals that some of them presented severe forms of HPE (alobar HPE/cyclopia) (Burrig et al., 1989; Chen et al., 1999; Kurtzman et al., 1987). However, these were the cases that carried an unbalanced translocation and the fetus that we report here had a pure terminal deletion.

Patient 3 carried a 7q36.1 deletion along with the duplication of Xq28. Xq28 duplication is known to cause developmental delay, distinctive facial features, major axial hypotonia, severe feeding difficulties, abnormal genitalia and susceptibility to infections (Sanlaville et al., 2005). Like other two patients, she did not present with HPE and sacral agenesis. It is apparent that the deletion of 7q and the genes *SHH* and *HLXB9*, did not have any severe effects on the phenotype in patients 2 and 3, even though it was a larger deletion than in patient 1. Other symptoms like microcephaly, growth and mental retardation, hypotonia, hypotelorism, urogenital anomalies are common to most of the chromosomal deletions (Evers et al., 1996; Hiraki et al., 2008; Scigliano et al., 2004; Telford et al., 1990) signifying the requirement and role of many genes present on different chromosomes in the normal development of the human brain.

We have discussed our three new patients (two 7q36 (m, o) + one 7q35 (n)) and twelve previous cases (ten 7q36 + two 7q35) from the literature that were not included in the review by Lukusa et al. (Lukusa et al., 2005) (table 2). The cases from table 2, 30 previous cases with 7q36 deletion and 10 cases with 7q35 deletion reviewed in 2005 (Lukusa et al., 2005) were combined in figure 5. In total there were forty-two 7q36 and thirteen 7q35 deletion cases. By summarizing the symptoms presented by these patients it was observed that growth retardation, microcephaly,

sacral malformation, HPE, large malformed ears, cleft lip and palate were the most common among them. Other symptoms that were observed in a few patients were- psychomotor delay, feeding problems, hypotonia, hypotelorism, downslanting palpebral fissures, midfacial hypoplasia, micrognathia, depressed nasal bridge, recurrent urinary tract infection, single central maxillary incisor etc.

While sacral malformations were common to cases with both pure terminal deletions and unbalanced translocations (A, B), HPE was mostly present only in cases with unbalanced translocations (Figure 5). Out of the 13 cases that presented with HPE only one case had a pure terminal deletion (C, D). Even though patient 3 had an unbalanced translocation, this pattern was not observed in her probably due to the small length of the duplicated segment Xq28, rendering it inconsequential. Furthermore, both HPE and sacral agenesis together were found in only four cases out of the 55 reported. Of these four cases, three were with unbalanced translocation and one with pure terminal deletion (E and F). This implies that unbalanced translocations can manifest into more severe phenotypes. In such patients, for example patients b, c, i and k (table 2), the over expression of a gene due to partial trisomy of the chromosome involved could be the cause of variable phenotypes. However in cases of pure 7q terminal deletions, there can be many reasons of such variable phenotypes.

All of our three cases carried different lengths of deletion. In patient 1, the array results demonstrated that the deletion was rather terminal localized to 7q36.2. It is possible that the patient did not present any severe signs of HPE since this was not an "extensive" deletion. But

the patients 2 and 3 carried a more "extensive" deletion than patient 1, which would mean a more severe phenotype. However this was not the case, and only the patient with the smallest deletion expressed sacral agenesis (patient 1). As a result, the possibility of the extent of deleted segment related to the phenotype can be excluded, at least for these three cases.

Various mechanisms and hypothesis can be used to explain the heterogeneous symptoms.
It is unlikely that one single mechanism is accountable for phenotypic variations in all cases of 7q terminal deletion.

One hypothesis is the role of modifier genes as described in case of cystic fibrosis (CF) (Davies et al., 2005), where the severity of CF can vary in siblings carrying a similar mutation in the *CFTR* gene. All of our patients had microcephaly, which can be considered a minimal HPE manifestation. It can be assumed that some genetic modifiers of the gene *SHH* regulate its expressivity level through feedback mechanism and modify the product transcripted from the intact gene, in case of its deficiency.

Polymorphism(s) in the coding region of DNA has the ability to affect the response to drugs and to influence the severity of a disease. It is possible that protein deficiency resulting due to the deletion was compensated by the genes present on the other homologous chromosome, which in turn is determined by polymorphism. It appears that polymorphism plays a crucial role in patients manifesting different phenotypes in different individuals. It would also explain the familial inheritance of HPE or sacral agenesis. Polymorphism in the genetic modifiers of the

genes *HLXB9* or *SHH* might also be one of the causes. For example in our study, patients 2 and 3 did not present with sacral malformation while patient 1 did. Perhaps there was some kind of polymorphism present in the intact gene *HLXB9* of the other normal chromosome 7 which regulated efficiently the gene product in these two patients.

Another mechanism to explain variability of symptoms is "multiple hit hypothesis" proposed by Ming et al. (Ming et al., 2002). According to this hypothesis there are many factors besides a single gene, which can control the expression of certain phenotypes, like some genetic factors or environmental conditions. It is difficult to accurately determine these factors since their effects can take place at any stage beginning from the formation of gametes.

The presence of severe forms of HPE with or without sacral agenesis has been attributed to position effect in cases of balanced translocation involving chromosome 7 (Bedell et al., 1996; Fernandez et al., 2005; Kleinjan et al., 2005; Kleinjan et al., 1998). It has been proposed that a rearrangement like a balanced translocation may juxtapose a gene with an enhancer element from another gene leading to inappropriate gene expression. In other instances, a gene(s), through a translocation is placed in a heterochromatic region thereby inhibiting the expression of the gene. For example, even though patient d (table 2) with a balanced translocation presented with mild form of HPE and no sacral agenesis, she suffered from other severe symptoms due to two position effects (Fernandez et al., 2005).

CONCLUSION

Our work gives for the first time a very high-resolution characterization of 7q terminal deletion. An important observation that we made was that HPE was mostly found in unbalanced translocation cases. Thus, we can conclude that when a 7q terminal deletion is accompanied with a partial trisomy of some other chromosome it results in more severe malformation of the brain. This can be attributed to extra dosage of genes. HPE in association with sacral malformation was present only in ~7% of the total cases studied (table 3). Consequently, a combination of these two manifestations together is very rare, irrespective of the kind of rearrangement (pure deletion or unbalanced translocation) and/or the band deleted (7q36 or 7q35).

We propose that haploinsufficiency of the deleted genes were compensated by the product of the genes present on the intact homologue resulting in variable phenotypes. Whether it is compensated or not can be attributed to varying genetic makeup (polymorphism) in different individuals thus resulting in different phenotypes.

Therefore, we also assume that it is not just one gene which is responsible for a particular phenotype like HPE or sacral malformations but various other genes, familial factors and environmental factors play a role too. Thus, it appears like it is a "combined effect" rather than a single-gene effect.

Array-CGH enabled us to determine the genes lost. In future, it would be interesting to perform array-expression and study the proteins expressed in different patients that would help understand the wide range of phenotypes in these deletion carriers.

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Table 1: Microsatellite markers for the three patients.

Markers	Position	Patient 1	Patient 2	Patient 3
D7S2474	7p22.3	np	sp	sm
D7S2559	7p21.1	sp	ni	ni
D7S2251	7p14.2	+	+	ni
D7S691	7p14.1	sp	+	+
D7S527	7q21.3	+	sp	ni
D7S515	7q22.1	ni	ni	na
D7S640	7q32.3	na	+	+
D7S2452	7q33	sp	sm	ni
D7S2540	7q34	np	+	np
D7S661	7q35	+	+	ni
D7S2511	7q35	+	ni	ni
D7S688	7q36.1	np	np	+
D7S2426	7q36.1	+	-	ni
D7S636	7q36.1	+	-	ni
D7S642	7q36.1	np	np	+
D7S2439	7q36.1	np	np	ni
D7S483	7q36.1	+	-	-
D7S798	7q36.2	-	-	-
D7S2462	7q36.2	ni	ni	-
D7S2546	7q36.2	-	-	ni
D7S2447	7q36.2	-	ni	ni
D7S550	7q36.3	ni	ni	-
D7S3037	7q36.3	ni	np	-
D7S2465	7q36.3	-	ni	-
D7S559	7q36.3	-	ni	ni
D7S2423	7q36.3	-	np	-

np- not performed, na- not amplified, ni- non informative, sm- semi-maternal, sp- semi-paternal, + present, - deleted

Table 2: Details of the 7q36 and 7q35 patients not reviewed by Lukusa *et al*, and our three new patients.

Patient ID	Reference	Band	Inheritance pattern	HPE	Sacral agenesis
a	*(Su et al., 2008)*	46,XX,del(7)(q36)	*de novo*	no	sacral agenesis
b	(Ginocchio et al., 2008)	46,XY,der(7)t(3;7)(p26.3;q36.1)	maternal	no	no
c	*(Kanafani et al., 2007)*	46,XY,t(7;8)(q31.3;q12).ish, del(7)(q36), inv(7)(q31.3q3?6)der(8)t(7;8)(q31.3;q12)	*de novo*	semilobar HPE	no
d	*(Fernandez et al., 2005)*	46,XX,t(6;7)(p21.1;q36)	*de novo*	Mild form	no
e	*(Horn et al., 2004) (patient 1)*	46, XY, del(7)(q36.3)	*de novo*	no	Partial sacral agenesis
f	*(Horn et al., 2004) Patient 2*	46, XY, del(7)(q36.3)	*de novo*	no	no
g	*(Horn et al., 2004) Patient 3*	46, XY, del(7)(q36.3)	*de novo*	no	no
h	*(Horn et al., 2004) Patient 4*	46, XY, del(7)(q36.3)	*de novo*	no	no
i	*(Le Caignec et al., 2003)*	46,XX/46,XX,add(7q36).ish der(7)(q36),t(2;7)(p22;q36)	*de novo*	no	sacrococcygeal teratoma
j	(Benzacken et al., 1997) (case 1)	46,XX.ish,del(7q36)	*de novo*	semilobar HPE	S1and S2 hemivertebrae
k	(Benzacken et al., 1997) (case 4)	46,XX,der(7)t(1;7)(q41;q35)	maternal	alobar HPE	partial sacral agenesis
l	(Roessler et al., 1997b) Patient 28	46,XX,del(7)(q35)	-	no	Sacral hypoplasia
m	Patient 1 (this study)	46,XX,del(7q36.2)	*de novo*	no signs	partial sacral agenesis
n	Patient 2 (this study)	46,XY,del(7q35)	*de novo*	no signs	no
o	Patient 3 (this study)	46,XX,der(7),t(X;7)(q28;q36.1)	*de novo*	no signs	no

FIGURES AND LEGENDS

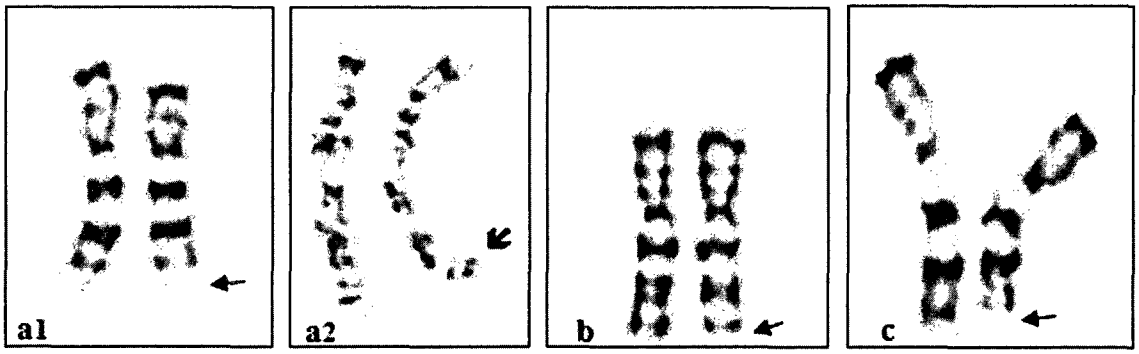

Figure 1: Low-resolution (a1) and high-resolution (a2) karyotypes of the patient 1, low-resolution karyotype of patient 2 (b) and patient 3 (c) showing the terminal deletion (indicated with an arrow).

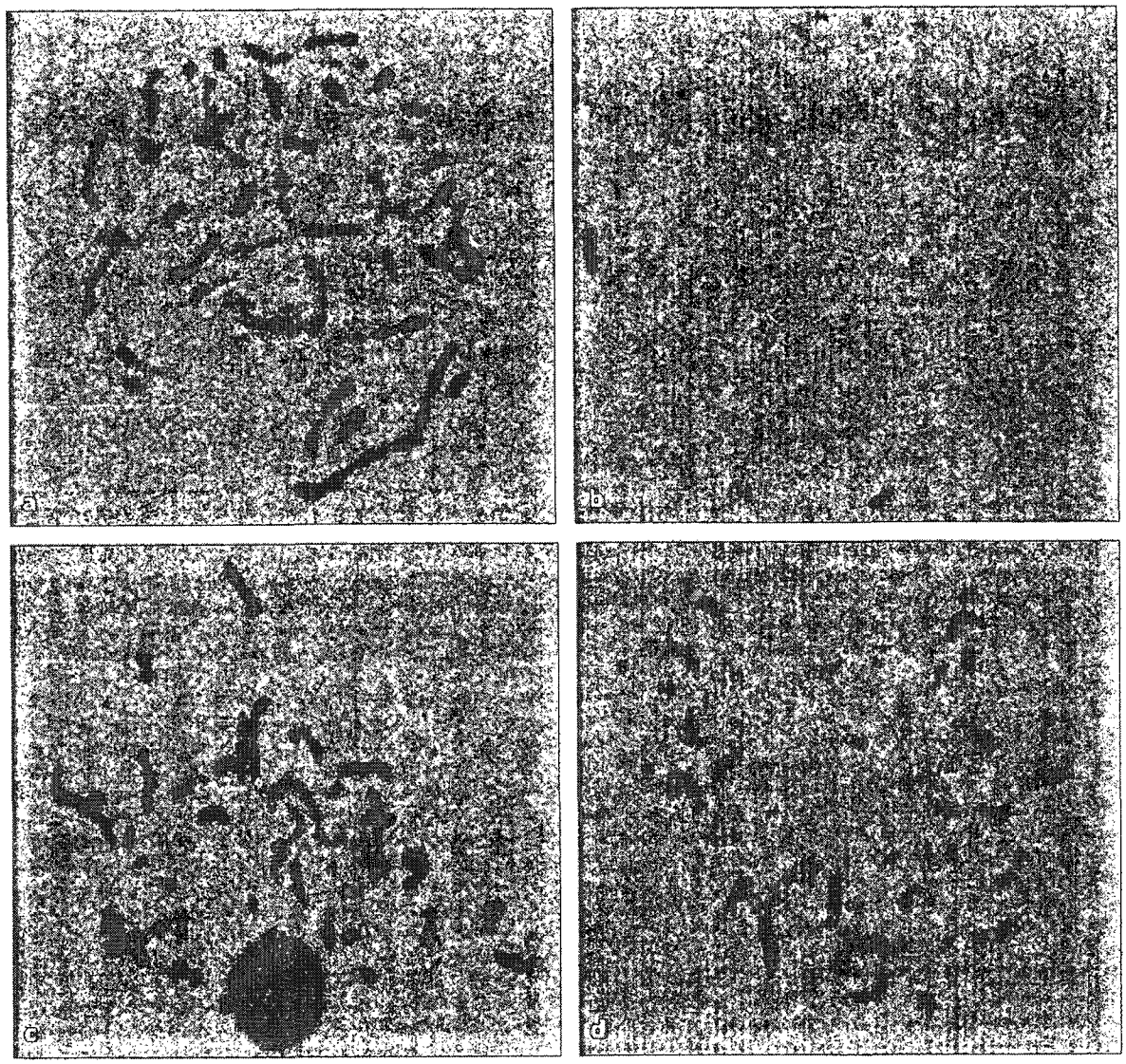

Figure 2: FISH with sub-telomere, 7p in Spectrum Green and 7q in Spectrum Orange for chromosome 7 on metaphase in patient 1 (a), patient 2 (b), patient 3 (c), Yellow 14q* and Aqua 14q11.2* (locus VIJyRM2000, Vysis) (c), CEP X in Aqua, Xq/Yq in Yellow, 2p* in Green and 2q* in Red, Xq duplication confirmed in patient 3 (locus Cdy16c07, Vysis) (d).

* The extra probes on chromosome 2 and 14 were present in the commercially purchased probe mix that was used for chromosome 7 and X, and were not hybridized on purpose.

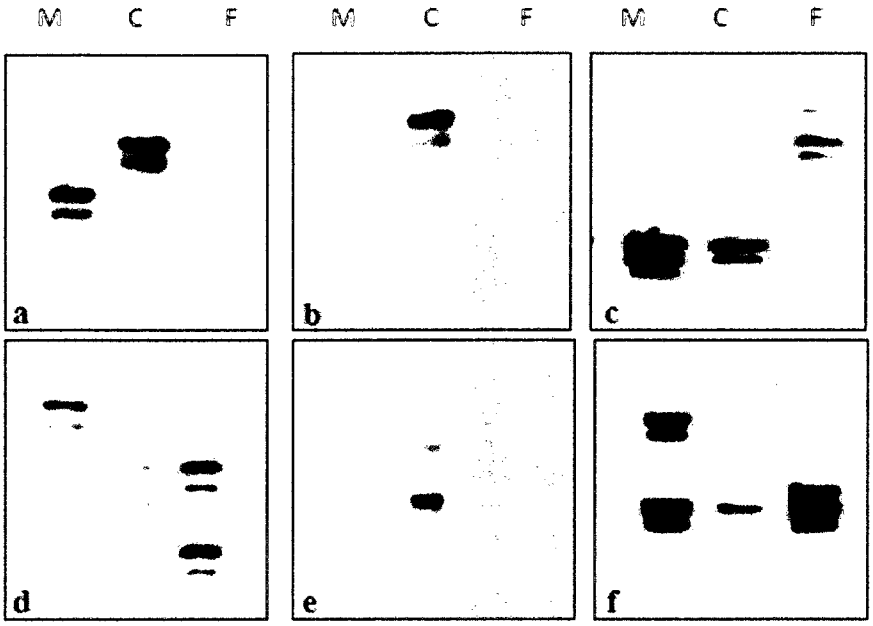

Figure 3: Example of deleted markers, D7S2465 in patient 1 (a), D7S798 in patient 2 (b) and D7S483 in patient 3 (c).
Non-deleted markers, D7S483 in patient 1 (d), D7S661 in patient 2 (e) and D7S688 in patient 3 (f).

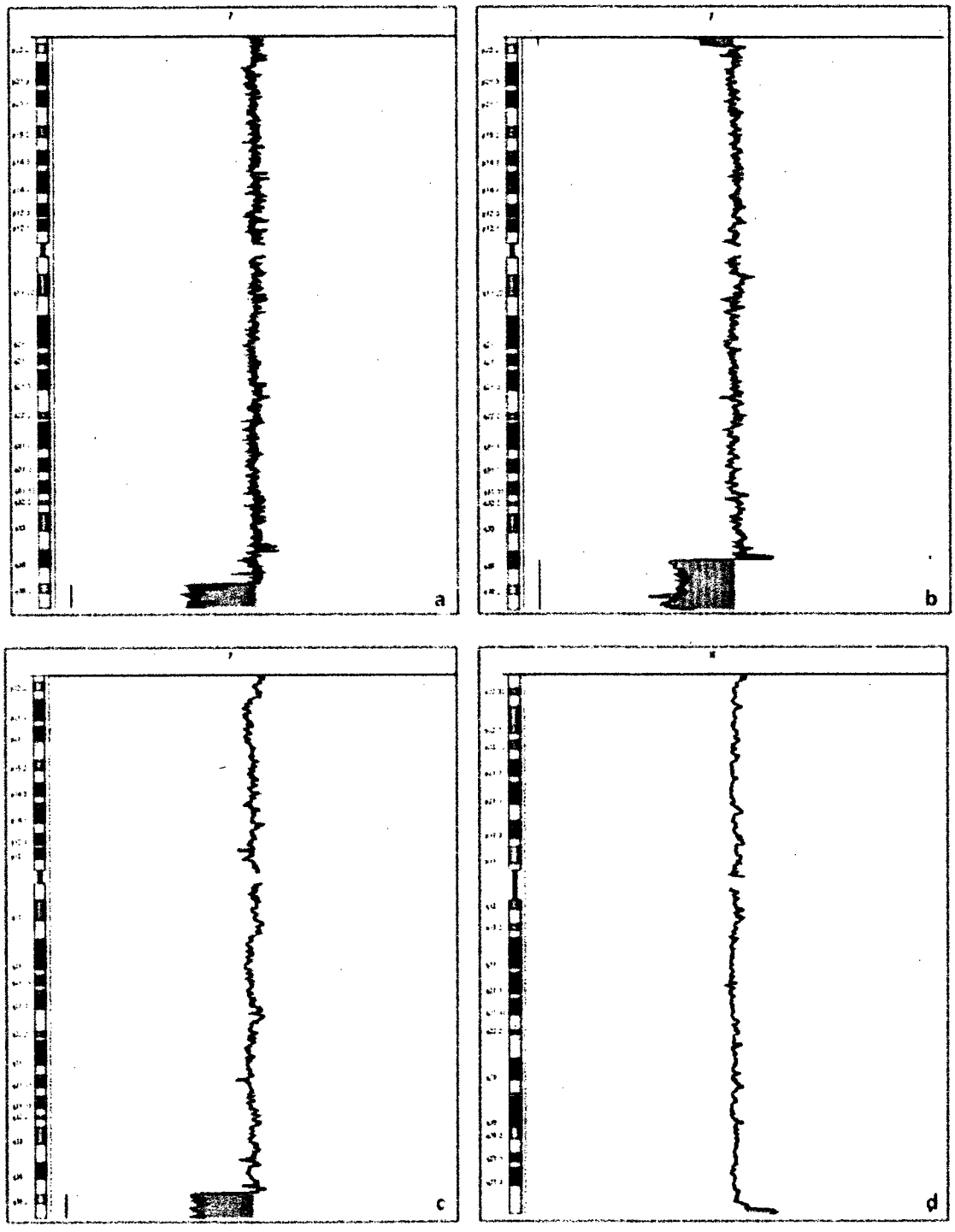

Figure 4: Array-CGH analysis depicting 7q36.2 and 7q35 deletions in patients 1(a) & 2(b) respectively, and patient 3 depicting deletion 7q36.1(c) along with duplication Xq28 (d).

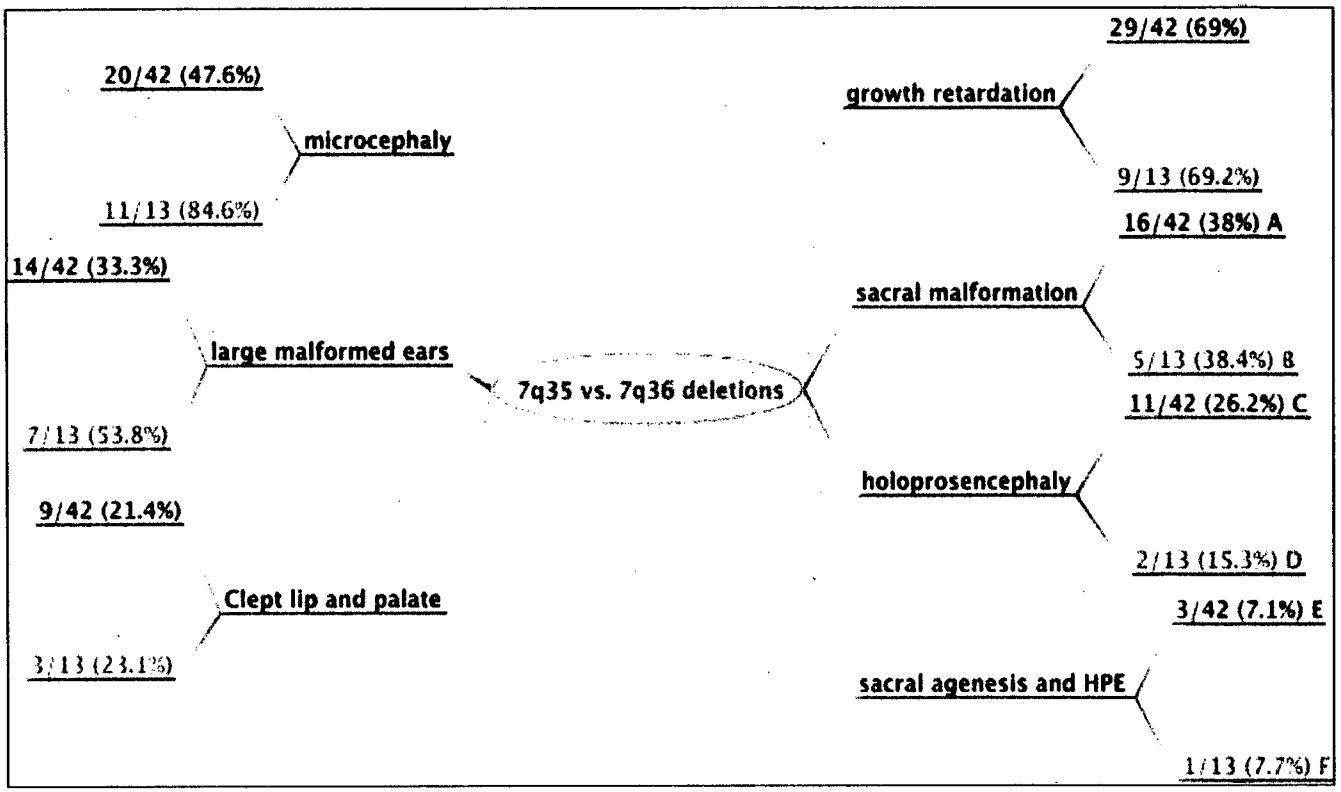

Figure 5: Main clinical symptoms presented by 7q35 (in green) and 7q36 (in blue) deletion patients. A- 9 cases with P.d*, 7 with U.t**; B- 3 cases with P.d, 2 with U.t; C- 1 case with P.d, 10 with U.t; D- both cases with U.t; E- 1 case with P.d, 2 with U.t; F- 1 case with U.t.

* **P**ure terminal **d**eletion
** **U**nbalanced **t**ranslocation

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GENERAL DISCUSSION

Chromosomal deletions account for around 1-4.5% of all detected chromosomal abnormalities (Forrester et al., 2007). We reported three patients with 7q terminal deletion and reviewed previously published cases. The symptoms resulting from such deletions come under the "7q deletion syndrome" (Harris et al., 1977). The breakpoints for this syndrome have been variable and can range from 7q32, 7q33, 7q34, 7q35 to 7q36. We have confined our study to patients with 7q35 and 7q36 deletions.

Determination of breakpoint

Patient 1 presented developmental delay, microcephaly, dysmorphic signs, partial sacral agenesis and moderate hearing problems.

Patient 2 was an aborted male fetus, 22 weeks old. Apart from slight microcephaly and pylectasia of the right kidney, there were no apparent malformations. No signs of HPE or sacral agenesis were observed.

Patient 3 who was a 2 yr and 8 months old girl, on examination showed signs of delayed development and microcephaly. No other severe signs were observed, she was relatively normal.

Consequently, their chromosomes were investigated. The phenotype in patient 1 was ascribed to the terminal deletion of the long arm of chromosome 7 i.e. deletion 7q36.2-qter, which is relatively a rare deletion. This deletion was not detected using standard low-resolution karyotype and could only be seen using a high-resolution karyotype. This

case highlights the importance of higher resolution karyotyping in cases carrying smaller deletions or rearrangements, which often go unnoticed using conventional standard cytogenetics. Using sub-telomere FISH specific for chromosome 7, we were able to confirm the result of high-resolution karyotype.

Similar procedure was followed for evaluating the patients 2 and 3, by performing karyotype and then sub-telomere FISH as described above. It was observed that both of these cases carried 7q terminal deletion.

However, there is a limitation to these techniques and through them we were unable to determine the breakpoint and whether it was present on maternal or paternal chromosome. Therefore we used higher resolution techniques like microsatellite analysis and aCGH to further localize the breakpoint.

By microsatellite analysis we were able to determine that the deletion was on the maternally derived chromosome in patient 1 and on paternally derived chromosomes in patients 2 and 3.

In patient 1 the breakpoint roughly lay between the markers D7S483 at 151.83 Mb at 7q36.1 and D7S798 at 152.44 Mb at 7q36.2. Consequently, it was not possible to know whether the deletion started at band 7q36.1 or 7q36.2, as a result determination of all the genes lost became difficult. Array-CGH analysis was performed to look for cryptic aberrations. No other anomalies were found. The result revealed the deletion started not at 7q36.1 but at 7q36.2. Thus the patient was monosomic for region 7q36.2.

In patient 2 the breakpoint lay between the markers D7S661 at approximately 143.20 Mb at 7q35 and D7S2426 at 149.10 Mb, band 7q36.1. Again the exact band at which the

deletion started could not be known. This was followed by aCGH to look for cryptic aberrations and to locate the exact band deleted. The deletion started at band 7q35 and no other chromosomal anomalies were reported.

In patient 3 the deletion was localized between the markers D7S642 at approximately 150.58 Mb and D7S483 at 151.83 Mb, band 7q36.1. The band at which the deletion originated was determined (7q36.1) but there was an approximately 1 Mb difference between the two markers, thus, aCGH was performed. It was revealed that along with the 7q36 deletion, there was an Xq28 duplication too, which was missed by performing the standard karyotype. Since it was only a 1.2 Mb duplication, this size of anomaly is not visible using standard cytogenetics, signifying why it is important to perform aCGH and how it has been lately used as a discovery tool.

Mosaicism

A chromosome analysis of buccal smear and fibroblast cells was done in patient 1 (skin and buccal smear samples were not available from patients 2 and 3) to look for any possible mosaicism. Mosaicism is the presence of two populations of cells with different genotypes in an individual. Since the patient did not present any signs of HPE, it was hypothesized that she was probably mosaic for the deletion and that her cells other than blood did not have any deletion which compensated for the *SHH* protein loss in her blood cells. To verify this, sub-telomere FISH on her buccal smear cells and skin cells was performed specific for chromosome 7. It was observed that the deletion was also present in skin and buccal smear cells ***(Figure 18)***.

Figure 18: *FISH with sub-telomere probes 7q in SO, 7p in SG, on buccal smear and fibroblasts cells. Only one red signal confirms deletion 7q.*

Therefore, due to the deletion, a lot of genes were missing. Some of these genes are crucial, required during embryological development and are especially involved in neurological development. These genes were- *HTR5A, EN2, SHH, LMBR1, HLXB9,* and were found to be deleted in all the three cases.

Comparison with the literature

Various chromosomal deletions have been reported and described in the literature. Considering the cases carrying terminal deletion of the long arm of other chromosomes, it was observed that there are a lot of common malformations that are not chromosome specific but are more due to the deletion or mutation of the genes that these chromosomes carry. This pattern was also observed in cases with 1p36 and 14q terminal deletion as mentioned in the introduction. For instance, some of the symptoms that were common to

the three syndromes (1p36, 14qter and 7q35-q36) were- microcephaly, broad nasal bridge, hypotonia, mental retardation and developmental delay. A lot more symptoms were common between 1p36 and 7q35-36 deletions. In other words, various chromosomal deletions produce different but characteristic syndrome with some degree of overlap. Others common features that were noticed were, multiple congenital anomalies, psychomotor retardation, mental retardation, developmental delay, dysmorphism, CNS (central nervous system) anomalies, -agenesis/thin corpus callosum, hydrocephaly, hypotonia, dysphagia/feeding difficulties, microcephaly, midface hypoplasia, upslanting palpebral fissures, epicanthic folds, prominent forehead, hypertelorism, deep set eyes, broad nose/flat nasal bridge, downturned corners of the mouth, micro/retrognathia, cleft lip or palate, low set/dysplastic ears, clinodactyly of the 5th fingers, small hands, tapering finger, urogenital anomalies, minor cardiac malformation and/or moderate hearing loss (Evers et al., 1996; Hiraki et al., 2008; Kitsiou-Tzeli et al., 2007; Scigliano et al., 2004; Taysi et al., 1979; Telford et al., 1990) associated with the terminal deletion of 1q, 2q, 6q, 10q and 14q. As a consequence, the symptoms appear to be regulated by gene products of various chromosomes together during the embryonic development of a human.

Beside association with 7q36 deletion, HPE has been found in 70% infants with trisomy 13, but is also associated with trisomy 18 and triploidy (Gurrieri et al., 1993). The following chromosome anomalies are also non-randomly associated with HPE; del(18p), dup(3)(p24-pter), del(2)(p21), and del(21)(q22.3) (Gurrieri et al., 1993). Mothers with diabetes have an increased incidence of bearing a child with brain anomalies (Cohen 1989). Mutation of some genes such as ZIC2, SIX3, TGIF, PTCH, GLI2, FAST1,

TDGF1, and DHCR7 is also known to result in HPE (Cohen 2006).

However none of the cases that we present here had HPE and only one patient had right kidney pyelectasis. These three cases give an interesting insight into the "variability of symptoms" presented by different 7q deletion carriers. Apparently, the case with the smallest deletion (7q36.2-qter) (Case 1) had sacral agenesis. The case with the largest deletion (7q35-qter) (Case 2) among the three had minimal symptoms. And the 3rd case (Case 3) with a translocation did not present any severe symptoms too.

Lukusa and colleagues (Lukusa et al., 2005) reviewed 10 cases with 7q35 deletion and 30 cases with 7q36 deletion. Out of these 30 cases, nine presented with HPE (Borovik C. L. 1987; Burrig et al., 1989; Chen et al., 1999; Chen et al., 1996; Grass F.S. 1995) (patient 3), (Kleczkowska et al., 1990; Kurtzman et al., 1987; Morichon-Delvallez et al., 1993; Nowaczyk et al., 2000), all these patients carried an unbalanced translocation. Eleven cases had sacral malformations, five cases with pure terminal deletion (Frints et al., 1998) (patient 2), (Masuno et al., 1990; Petrusevska 1996; Rodriguez et al., 2002), and six cases with unbalanced translocations (Morichon-Delvallez et al., 1993; Nowaczyk et al., 2000; Savage et al., 1997; Wang et al., 1999). In the above cases, both HPE and sacral malformation in association were present in only two of them (Morichon-Delvallez et al., 1993; Nowaczyk et al., 2000).

For a total of 10 cases with 7q35 deletion, only one patient had HPE (Vance et al., 1998) (patient V-I); this case had an unbalanced translocation between chromosome 3 and 7

resulting in monosomy 7q35 and trisomy 3p22. Three patients presented with sacral malformation, two with pure terminal deletion (Masuno et al., 1996; Turleau et al., 1979) and one with an unbalanced translocation (Shaffer et al., 1996). HPE and sacral malformations together were not seen in any case.

A total of 15 cases are described in table 2, including our three cases. Three patients were with 7q35 deletion and twelve with 7q36 deletion. In 7q36 deletion patients, only two carried HPE, one with an unbalanced rearrangement (c) and another with pure terminal deletion (j). Sacral malformations were observed in five patients, four of which were pure deletions (a, e, j, m) and one was an unbalanced rearrangement (i). Together HPE and sacral anomalies were observed in one patient (j) only. Out of the three cases of 7q35 deletion, one presented with HPE (k), with an unbalanced translocation; two carried sacral malformations, one with pure deletion (l) and other with an unbalanced translocation (k). Patient k presented both HPE and sacral malformation in association.

Summarizing the data in figure 5 from Lukusa's review and table 2, we got forty two 7q36 and thirteen 7q35 terminal deletion cases (figure 5), a total of 55 cases. We observed that severe forms of HPE were found in patients with unbalanced translocation, meaning, monosomy of 7qter region and trisomy of terminal region of some other chromosome (figure 5: C, D). It was also observed that both sacral agenesis and HPE in association were present in less than 10% of the cases (E,F). Sacral malformation was observed in approximately 38% of the cases reported in both 7q35 and 7q36 deletion (A,B)

Severe phenotype in unbalanced translocation and pure terminal deletion cases was probably due to loss of genetic material (and may be extra dosage of genes in case of partial trisomy). A balanced translocation implies no loss or gain of genetic material, yet some cases had an abnormal phenotype ascribed to position effect. A minimal critical region for the locus *HPE3* (gene *SHH*) was refined by analyzing 34 cell lines (Roessler et al., 1997b). In some instances with patients carrying a balanced translocation between chromosome 7 and other chromosome, even when the gene *SHH* was intact i.e. the breakpoint was outside the putative gene, some cases did present HPE (Roessler et al., 1997b). This is termed as position effect. Position effect can be defined as a harmful change in the level of gene expression through long-range effects on chromatin structure or by disruption of regulatory elements.

However the cause of the variability of symptoms was unclear. The DNA sequence of two unrelated individuals is almost 99.9% identical. The rest of the DNA contains variations called as polymorphism. Polymorphisms in the coding region of DNA are of much importance and imply a significant function. It has the ability to affect the response to drugs and to influence the severity of a disease. There can be an existence of multiple alleles at a locus, each with a different effect on the phenotype. These alleles possess different mutations that alter the protein function, thus producing changes in phenotype.
It is also possible that there are some genetic modifiers of the genes *SHH* and *HLXB9* which alter the expression of these genes resulting in heterogeneous phenotypes. Modifier genes are the genes that affect the gene expression of the target genes. Polymorphism in

the modifier genes may also result in altered expression. Single Nucleotide Polymorphisms (SNPs) are single base pair positions in the genome at which variation in nucleotide gives rise to alternative alleles in normal individuals and populations (Brookes 1999). An SNP in the promoter region of the modifier gene will affect the transcription rate, in an exon will affect the protein structure or function, and an SNP within the intron will interfere with the splicing (Brookes 1999).

Conclusion

Utilizing various conventional and molecular cytogenetic techniques we were able to extensively and accurately characterize the deletion in our patients. It was performed in a step-by-step manner, advancing from low-resolution to high-resolution analysis. This was followed by a careful and thorough review of the literature with 7q terminal deletion cases which assisted us in resolving the apparent cause of the variable phenotypes.

We propose a possible existence and role of genetic modifiers along with polymorphism and various environmental factors as an explanation for this kind of pattern. In some cases, perhaps the gene product is balanced by the expression of the genes from intact homologous chromosome resulting in milder phenotype.

An extensive study in this regard including a large number of patients is required to validate this theory. It is important to note here that the evaluation of chromosome 7 will be helpful in patients who show signs of caudal agenesis or HPE, even though these symptoms were not very frequently seen. Furthermore, a high-resolution analysis like

aCGH should be encouraged for cases with miscellaneous symptoms where there is no previous knowledge of the anomaly.

Patients with an unbalanced translocation involving monosomy of 7qter region should be evaluated more carefully since they are more likely to present with severe forms of HPE. Even though sacral agenesis and HPE have always been linked with 7qter deletion, we found that these symptoms were present in association in only less than 10% of the cases.

The next step in this regard would be to perform array-expression in future cases with this deletion to study polymorphism and protein products. Further research is likely to improve our understanding on how certain genetic modifiers and polymorphisms result in heterogeneous phenotypes, along with the effects of "gene-environment interactions".

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